THE GIZZARD FACTOR OF THE CHICK

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ONE FIGURE

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It has been demonstrated (Dam, '35 a, b; Almquist and Stokstad, '35 a, b; Almquist, '36; Dam and Schonheyder, '36) that a hemorrhagic disease of chicks is due to the lack of a new fat-soluble vitamin. In these investigations erosions of the gizzard lining have been frequently found but their occurrence has not been closely correlated with the hemorrhagic syndrome, since levels of anti-hemorrhagic vitamin supplements which have sufficed to prevent hemorrhages and delayed blood clotting have not prevented gizzard erosions. Gizzard erosion has been commonly noted in the routine pathological examination of chickens given the usual practical diets (Jungherr, '35). Such erosions have been observed at this laboratory in embryos in late incubation stages and in day-old chicks (Holst and Halbrook, '33). On the other hand, the hemorrhagic syndrome has not been reported in chicks reared under practical conditions.

This gizzard disorder of chicks is characterized by erosion or necrosis of the secreted gizzard lining, usually at the cardiac end of the gizzard but often in other portions. The erosions frequently are confined to the surface of the lining but may be severe enough to penetrate the epithelium and the muscular gizzard walls. The lining near the erosion is frayed and loosened in most cases and may be brown or black colored. The unaffected lining is usually quite coarsely ridged. In view of the lack of evidence to classify these erosions as a

distinct part of the hemorrhagic syndrome, or even to demonstrate clearly that they are the result of a dietary deficiency, further investigations have been made. A preliminary report of this work has already been given (Almquist and Stokstad, '36). Since the latter report, there has appeared a paper by Kline, Bird, Elvehjem and Hart ('36) on 'vitamin B_4' studies in which it is stated that dried hog lung tissue is an effective supplement for the prevention of gizzard lesions. Attempts to extract the factor were unsuccessful. These authors state that lack of the substance preventing occurrence of lesions in the gizzard has a profound effect upon the growth of chicks. They also advance the opinion that gizzard lesions may contribute to poor absorption.

METHODS AND RESULTS

The methods and procedure were the same as described in a previous paper (Dam and Schonheyder, '36). White Leghorn chicks were fed a basal diet composed of ether-extracted fish meal 17.5 parts, ether-extracted dried brewer's yeast 5.0 parts, ground polished rice 75.5 parts, cod liver oil 1 part and salt plus small amounts of ferrous and cupric sulphates 1 part. This diet was designated basal diet D. That this diet was adequate for normal growth was proved by the fact that the average weights of chicks at 4 weeks and at 6 weeks were frequently above the usual weight standards, even in lots deficient in the anti-hemorrhagic factor. The external appearance and development of all lots were likewise excellent. Mortality not associated with some phase of the hemorrhagic disease was a rarity. Chicks were reared for a standard period of 4 weeks, except for those chicks given the basal diet only, in which case the birds usually all died of the hemorrhagic disease in less than 4 weeks.

At the end of the period birds were killed and the gizzards removed and opened. An arbitrary standard for scoring the severity of the gizzard erosion was employed, a single plus sign representing a distinct erosion, two plus signs a severe erosion and three plus signs a very severe and wide-spread erosion. Doubtful cases were given a value of one-half plus.

The results of several preliminary experiments demonstrated conclusively that gizzard erosion was alleviated by high levels of a hexane extract of alfalfa and by whole alfalfa. A progressive decrease in severity of erosions was found as the dosage of the alfalfa hexane extract was increased by steps up to 25% equivalent, at which level complete or nearly complete prevention of erosions was obtained. Whole alfalfa at a 10% level did not prevent distinct erosions. At a 20% level of alfalfa, the erosions were much less severe but not completely prevented. The alfalfa hexane-extract was shown by separate assay with chicks to have an anti-hemorrhagic potency equivalent to that of the dried alfalfa from which it was prepared. The dosage of this extract required to prevent gizzard erosion was approximately 100 times the dosage required to prevent hemorrhages.

Certain other dietary supplements were tested for their influence both on hemorrhages and on gizzard erosions in an attempt to find a supplement which would prevent the latter without affecting the former. Results of these experiments are given in table 1.

From the data of table 1 it is evident that supplements rich in the known vitamins, fat and phospholipids failed to prevent gizzard erosions. Many of the supplements which prevented hemorrhages had no noticeable effect on gizzard erosions. On the other hand, the birds fed the fresh kale were entirely free from gizzard erosions. Dam ('35) has published data on a few birds fed orange meal which developed hemorrhages but no gizzard erosions. The above experiments with orange oil and with orange meal have differed from Dam's result.

A further series of experiments was conducted for the purpose of comparing the potencies of alfalfa, kale and hempseed extracts in preventing hemorrhage and gizzard erosion. The basal diet was slightly modified to the following composition: ether-extracted fish meal 17.5 parts, ether-extracted brewer's yeast 7.5 parts, ground polished rice 73.0 parts, salt mixture 1.0 part and cod liver oil 1.0 part, and was designated diet E. The procedure followed was the same as already described. The results of these experiments are given in table 2.

TABLE 1

Effect of certain dietary supplements on hemorrhages and gizzard erosions in chicks at 4 weeks of age

SUPPLEMENT TO DIET D	MANNER OF FEEDING	NUMBER OF CHICKS	EFFECT ON HEMORRHAGES	AVERAGE NUMBER OF + SIGNS PER GIZZARD
Cod liver oil	5% of diet	7	Negative	0.86[1]
Wheat germ oil, highly potent in vitamin E	5% of diet	10	Negative	1.40[1]
Orange oil	0.5% of diet	10	Negative	1.12[1]
Orange meal	5% of diet	10	Marginal	1.30[1]
Dried skim milk	5% of diet	10	Marginal	1.20
Chicken liver, vacuum dried, from chickens on normal diets	10% of diet	15	Marginal	0.80[1]
Liver extract[2]	100 cc. per kilogram of diet	8	Negative	1.10[1]
Fresh lemon juice	2 cc. orally per bird per day	11	Negative	0.96[1]
Egg yolk, hard boiled	One-fifth yolk per chick per day	15	Preventive	1.21
Egg white, hard boiled	One-fifth white per chick per day	15	Negative	1.10[1]
Fresh yellow carrots, no green portions used	ad. lib.	15	Negative	1.20[1]
Water extract of alfalfa	Equivalent to 10%	13	Negative	0.92[1]
Fresh kale	ad. lib.	11	Preventive	0.00
Hexane extract of alfalfa treated with magnesium oxide[3]	Equivalent to 40%	10	Preventive	1.10
Non-saponifiable alfalfa lipids	Equivalent to 40%	8	Preventive	1.28

[1] Several birds in this pen died with hemorrhages before 4 weeks. The gizzard scores for such birds have been included.

[2] Water extract of beef liver. One hundred cubic centimeters are equivalent to 1.7 kg. of whole liver. Kindly furnished by Dr. S. Lepkovsky.

[3] This treatment does not remove the anti-hemorrhagic vitamin.

Separate assays of the kale and alfalfa extracts showed that they were of nearly equal anti-hemorrhagic activity with the alfalfa extract being slightly more active than the kale extract. In comparison, hempseed extract contained little of the anti-hemorrhagic vitamin since it did not impart complete protection at a 5% equivalent level.

TABLE 2

Comparative potencies of alfalfa, kale and hempseed hexane extracts in preventing hemorrhages and gizzard erosions

SUPPLEMENT TO BASAL DIET E	EQUIVALENT PER CENT	NUMBER OF CHICKS	AVERAGE CHICK WEIGHT IN GRAMS	INCIDENCE OF HEMORRHAGES, PER CENT OF BIRDS	AVERAGE NUMBER OF + SIGNS PER GIZZARD	TOTAL MORTALITY, PER CENT OF BIRDS
None	..	8	....	100.0	0.94	100.0[1]
Alfalfa extract	¼	8	198.8	0.0	1.10	0.0
Alfalfa extract	½	8	186.2	0.0	0.85	0.0
Alfalfa extract	20	8	204.4	0.0	0.29	0.0
Alfalfa extract	25	8	197.5	0.0	0.13	0.0
Kale extract[2]	¼	8	196.5	0.0	0.44	0.0
Kale extract	½	8	201.3	0.0	0.31	0.0
Kale extract	5	8	190.0	0.0	0.19	0.0
Kale extract	15	8	201.3	0.0	0.06	0.0
Kale extract	25	8	221.2	0.0	0.06	0.0
Hempseed extract[4]	¼	8	203.4	62.5	1.10[3]	62.5
Hempseed extract	½	8	205.0	75.0	1.40[3]	50.0
Hempseed extract	5	8	197.3	12.5	1.00[3]	12.5
Hempseed extract	15	8	215.2	0.0	0.44[3]	12.5
Hempseed extract	25	8	210.0	0.0	0.25	0.0

[1] All birds died with hemorrhagic symptoms in less than 14 days.

[2] Prepared by thorough hexane extraction of vacuum-dried kale. Equivalent per cent refers to dried kale.

[3] Gizzard scores for birds which died have been included.

[4] Prepared by thorough hexane extraction of ground hempseed. Equivalent per cent refers to whole hempseed.

From the gizzard erosion standpoint, the kale extracts appeared relatively more active than the alfalfa extracts. The kale extract at a 5% equivalent level was about as effective in preventing erosion as the alfalfa extract at 25%. At lower dosages, the kale extract was consistently more effective on erosions than the alfalfa extract. Further trials at 5% equivalent levels of each extract gave similar results. The mortality

on the lower levels of hempseed extract was so severe that comparisons cannot safely be made. There was a decided reduction in severity of gizzard erosion at the higher levels of hempseed extract.

The amount of alfalfa extract required to prevent erosions as compared to that required to prevent hemorrhages was in a ratio of at least 100 to 1, respectively, while the corresponding ratio for the kale extract was approximately 20 to 1. The results obtained with the kale and alfalfa extracts, in particular, indicated that the fat-soluble factor influencing gizzard erosion was not the anti-hemorrhagic vitamin since these extracts, although equal in anti-hemorrhagic activity, were of distinctly different potency in preventing gizzard erosion. The fact that the factor which alleviated gizzard erosion was not in the non-saponifiable fraction or in the filtrate from the activated magnesium oxide treatment (table 1) constituted a further proof of its difference from the anti-hemorrhagic vitamin.

Experiments were next conducted to determine if this factor could be detected in the saponifiable lipid fraction. For this purpose chicks were kept on the basal diet for 1 week, then two groups of chicks were given preparations of kale lipids, one the non-saponifiable fraction equivalent to 20% dried kale and one the saponifiable fraction at the same dosage. A third group was given 1% equivalent of alfalfa hexane extract. After 2 weeks of such feeding, the birds were killed and the gizzards examined as before. The group receiving the non-saponifiable kale lipids had an erosion score of 1.10, that receiving the saponifiable kale lipids a score of 0.19, and that receiving the alfalfa extract 1.13. The gizzard score of 0.19 in the group fed the saponifiable fraction was due to three doubtful cases which probably were in the process of healing. This experiment showed that the anti-gizzard-erosion factor was not in the non-saponifiable fraction and was definitely present in the saponifiable fraction.

It was also found that, in contrast to the anti-hemorrhagic vitamin (Dam, '35 a, b; Almquist and Stokstad, '35; Almquist,

'36), the gizzard factor was destroyed readily by heat. Preparations of dried kale which were heated for 24 hours in a vacuum dryer at more than 100°C. completely lost their power to prevent gizzard erosion. The anti-gizzard-erosion potency of the hexane extracts was also adversely affected by saponification and only the most careful treatment during saponification would preserve some of the potency.

TABLE 3

Effect of certain supplements on gizzard erosions in the presence of the anti-hemorrhagic vitamin

SUPPLEMENT TO DIET E PLUS THE ANTI-HEMORRHAGIC VITAMIN	MANNER USED	NUMBER OF CHICKS	AVERAGE NUMBER OF + SIGNS PER CHICK
Wesson oil	5% of diet	16	0.85
Corn oil	10% of diet	8	1.00
Linseed oil	5% of diet	10	0.65
Hempseed oil	5% of diet	10	0.65
Walnut oil	5% of diet	10	0.60
Lard	5% of diet	10	0.85
None		10	0.95
Hog liver, air dried, 35°C.	5% of diet	9	0.72
Hog lung, air dried, 35°C.	7% of diet	10	0.85
Hog kidney, air dried, 35°C.	3% of diet	11	0.82
Hog heart, air dried, 35°C.	4% of diet	10	0.85
Hog pancreas, air dried, 35°C.	1.5% of diet	10	0.80
None		10	0.88
Hog lung, vacuum dried	5% of diet	10	1.06
None		10	1.11
Alfalfa ash	Equivalent to 25%	9	0.91
Cotton pulp	5% of diet	9	1.13
Calcium carbonate	5% of diet	9	0.94
Dried skim milk	5% of diet	15	0.93
Dried cabbage	5% of diet	9	0.90
None		12	1.02

The next experiments were designed principally to test for other sources of the gizzard factor using diet E supplemented with a plentiful amount of the anti-hemorrhagic vitamin. This was supplied in the form of kale or alfalfa hexane-extract treated with activated magnesium oxide and at a level equivalent to 2% of the dried leaf tissue. Results of these experiments are given in table 3.

The information summarized in table 3 indicates some slight preventive action in several of the oils but not sufficient to establish any of these oils as a practical supplement. The various air-dried hog tissues gave results not appreciably different from the control group. The vacuum-dried hog lung was also ineffective although prepared in a manner similar

TABLE 4

Effect of wheat bran, wheat bran hexane extract and alfalfa hexane extract on gizzard erosions

	GROUP			
	A	B	C	D
Diets				
Fish meal	17.5	17.5	17.5	17.5
Ground polished rice	58.0	73.0	58.0	58.0
Brewer's yeast	7.5	7.5	7.5	7.5
Wheat bran	15.0	...	...	15.0
Hexane extract of wheat bran, equivalent level	...	30.0	...	...
Wheat bran, hexane extracted	...	...	15.0	...
Salt mixture	1.0	1.0	1.0	1.0
Cod liver oil	1.0	1.0	1.0	1.0
Wesson oil	2.0	2.0	2.0	2.0
Alfalfa hexane extract treated with activated magnesium oxide, equivalent level	2.0	2.0	2.0	...
Alfalfa hexane extract, equivalent level	...	...	...	20.0
Number of chicks	9	9	9	10
Mortality	0	0	0	0
Average weight of chicks at 40 days in grams	318.0	317.5	334.0	319.2
Average number of + signs per gizzard	0.67	0.25	1.25	0.10
Texture of gizzards	Fine	Coarse	Fine	Fine
Average weights of gizzards in grams	8.9	7.5	9.3	8.6

to that of Kline, Bird, Elvehjem and Hart ('36). Growth was practically equivalent in all comparable lots.

Experience with practical chick diets led to the observation that diets containing wheat bran in addition to the dried alfalfa were productive of very slight erosions, if any. We accordingly investigated wheat bran. Results of an experiment in this connection are given in detail in table 4.

In this experiment chicks were raised to 12 days of age on diet E plus an adequate supply of the anti-hemorrhagic vitamin. A sample of these 12-day-old chicks was examined for gizzard erosion and found to have an average score of 1.06 (fifteen birds). Four groups of the most vigorous remaining chicks were selected and placed on diets as listed in table 4. They were kept on these diets for 4 weeks.

Inspection of these data shows that growth was equally good in all lots irrespective of the condition of the gizzards. Similar results are shown in table 2. Group A fed the whole wheat bran alone had definitely better gizzards than group C fed the extracted wheat bran. Group C had the most severe erosions, representing a complete lack of curative ability in the diet. The results with group B showed that the curative effect of wheat bran was localized in the hexane-soluble fraction. When whole bran was reinforced by a hexane extract of alfalfa, as in group D, nearly perfect gizzards resulted. The small scores in groups B and D are due to a few cases in which healing was not complete but formation of new lining at the location of the erosion was apparent.

The gizzards obtained from groups A, C and D were all well packed with fiber while those of group B were soft and flabby. The erosion score showed no relation to the presence of fiber or bulk in the diet, in agreement with results we have obtained with cotton pulp (table 3). Groups A, C and D exhibited a finely ridged texture and group B a coarsely ridged texture. Group B had the smallest gizzards. These characteristics also appeared unrelated to the erosions. We have made other observations which confirm these findings. Representative gizzards from these groups are illustrated in figure 1.

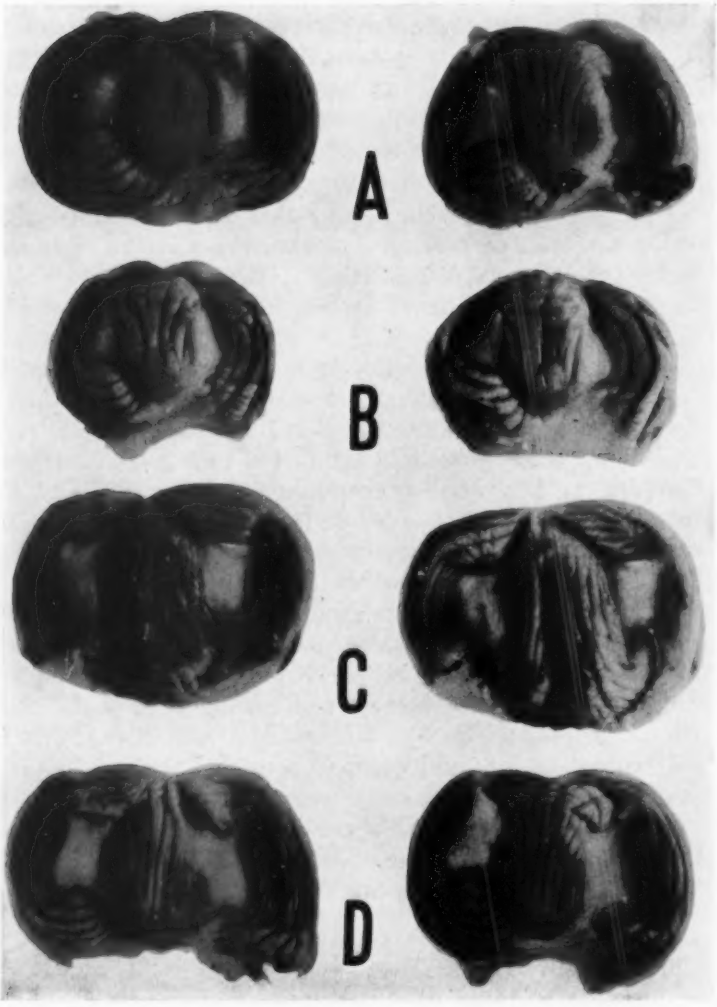

Fig. 1 Interior views of representative gizzards from group A—whole wheat bran, group B—wheat bran hexane extract, group C—hexane-extracted wheat bran, and group D—whole wheat bran and alfalfa hexane extract.

DISCUSSION

It has been shown in the preceding section that a comparatively unstable, fat-soluble factor is required by chicks for proper formation of the gizzard lining. Absence of this factor in the diet leads to erosions or lesions of the lining. Lesions can be prevented or cured by providing an adequate level of the gizzard factor. The factor can be clearly differentiated from vitamins A, D, E, F, the anti-hemorrhagic vitamin and the anti-encephalomalacic vitamin of Goettsch and Pappenheimer ('36). In comparison to the latter, the gizzard factor differs distinctly in occurrence, properties and pathology. At the present time, fresh or dried greens and wheat bran seem to be the best practical sources.

All of our data and observations lead to the conclusion that the gizzard factor is not a growth factor. Jukes ('37) has similarly observed that gizzard lesions do not affect growth. We are unable to explain the better growth observed by Kline, Bird, Elvehjem and Hart ('36) in birds given dried hog lung tissue as a preventive for gizzard lesions. It is possible that these workers may have unwittingly rectified their diets in respect to some other factor by the use of dried hog lung. Contrary to their report, we find no anti-gizzard-erosion activity in this supplement.

SUMMARY

1. Gizzard erosions or lesions of the chick are caused by a deficiency in the diet of a fat-soluble factor. This factor is not identical with any of the known vitamins.

2. The gizzard factor appears to be comparatively unstable being easily destroyed by heat and by alcoholic potash. It is readily adsorbed from solution in hexane by activated magnesium oxide. It appears to be in the saponifiable fraction.

3. The best practical sources of this factor yet found are fresh and dried greens and wheat bran.

4. Presence or absence of gizzard erosions or of the gizzard factor in the diet has no appreciable effect on the growth of chicks.

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THE EFFECT OF LOW FAT DIETS ON SERUM LIPIDS OF RATS[1]

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Although it is well known that the character of the body fats may be changed by dietary measures, very little definite information is available to indicate that the blood lipids may be similarly affected. Hansen and Burr ('33) observed that the serum lipids of rats fed on fat-free diets were of a lower degree of unsaturation than those of control animals on stock diet. Similar results were obtained by Williams and Maynard ('34) in their lactation studies on goats. Recent investigations by the authors indicate that the same phenomenon occurs in the human subject. The present report concerns a more detailed study of the relation between the diet and the serum lipids.

Female animals of the Wistar strain were used. The conditions as to environment and routine were the same as described previously (Burr and Burr, '29). Control rats were reared on the stock diet of McCollum and Simmonds ('18). This diet contains approximately 4.5% fat, of which the total extractable fat has an average iodine value of 58. In one group of animals on this diet, the food intake was so restricted as to keep their weight at the same level as that of the average of the fat-free rats of the same age. A second group of animals on the same diet was sacrificed at 2 months of age. The 550 B diet of Burr and Burr ('29) with a total fat content of less than 0.01% was used for the fat-free regimen. The ani-

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mals on this diet showed the typical findings of the fat deficiency syndrome. The esters of oleic acid and linolic acid were administered in various small quantities to several groups of animals on the fat-free diet.

Uniform fasting periods of 8 hours were used throughout the study. After mild chloroform anaesthesia, a small window

TABLE 1

Serum lipids of rats fed on stock diet

COLONY NO.	IODINE ABSORBED	CHOLESTEROL	TOTAL FATTY ACIDS	TOTAL LIPIDS	IODINE NUMBER OF TOTAL LIPIDS
	A. Normal adult rats				
	mg./100 cc.	*mg.%*	*mg.%*	*mg.%*	
147	325	108	193	301	108
10	431	111	280	391	111
15	428	116	271	387	111
470	548	84	306	390	140
13	308	74	209	283	109
246	554	113	367	480	115
219	384	74	246	320	117
338	446	60	275	335	133
472	409	74	287	360	114
11	277	68	174	242	114
12	320	71	200	271	118
41	478	118	292	410	117
39	438	102	256	358	123
23	410	80	265	345	119
14	482	79	321	401	120
Average	416	88.8	263	352	118
	B. Normal rats 2 months of age				
1	393	79	309	388	101
2	415	107	267	374	111
3	421	99	278	377	112
24, 25	422	103	265	368	115
Average	413	97	280	388	110
	C. Adult rats held to weight level of average of fat-free animals				
73	422	98	199	297	142
47	435	85	238	343	127
84	412	91	220	311	132
740	439	99	221	320	137
Average	427	93	220	318	134.5

was cut in the ventral thoracic wall and the heart was made to herniate. The heart was incised and the blood was collected directly into a test tube, gentle pressure being applied to the body to insure maximum collection of blood. For the most part the individual specimens were kept separate, however, at times large samples were obtained by pooling the blood from several animals of the same group. The blood

TABLE 2

Serum lipids of rats fed on the fat deficient diet

COLONY NO.	IODINE ABSORBED	CHOLESTEROL	TOTAL FATTY ACIDS	TOTAL LIPIDS	IODINE NUMBER OF TOTAL LIPIDS
	mg./100 cc.	*mg.%*	*mg.%*	*mg.%*	
330	262	84	212	287	91
308	330	75	252	337	98
55	269	87	285	372	72
73	267	78	239	317	84
77	267	86	232	318	84
63	431	129	360	489	88
74	329	81	282	363	91
75	374	84	285	443	84
67	343	92	335	377	91
155	423	140	283	423	100
136	330	100	218	318	103
65	282	93	200	293	96
53	393	110	290	400	98
56	308	98	211	309	100
59	328	68	231	299	109
55	269	87	285	372	72
73	267	78	239	317	84
77	267	86	232	318	84
60	530	106	437	543	97
Pooled (4)	305	84	274	358	85
Pooled (5)	310	66	300	366	85
Average	327.8	91.0	270.6	362.3	90.3

was allowed to clot, and the serum obtained by centrifuging for 20 minutes at 800 r.p.m. The alcohol-ether (3:1) extract was prepared immediately after the manner of Bloor. The cholesterol was determined on aliquots of the alcohol-ether extract using the Lieberman-Burchart reaction according to the method described by Bloor ('28). Aliquots of the original extract were saponified, acidified and extracted with petroleum

ether and the total fatty acids determined by the oxidative method of Bloor ('28). The iodine absorption value of the serum was obtained on aliquots of the alcohol-ether extract using the Rosenmund-Kuhnhenn ('23) method as modified by Page, Pasternak and Burt ('30). Yasuda's technic was followed except that N/100 instead of N/20 sodium thiosulphate

TABLE 3

Serum lipids of rats fed on the fat deficient diet plus esters of oleic and linolic acid

COLONY NO.	IODINE ABSORBED	CHOLESTEROL	TOTAL FATTY ACIDS	TOTAL LIPIDS	IODINE NUMBER OF TOTAL LIPIDS
	A. Methyl oleate 13 drops daily				
	mg./100 cc.	*mg.%*	*mg.%*	*mg.%*	
110	394	82	295	347	113
115	464	113	411	524	89
116	637	130	432	562	113
109	461	98	344	442	104
111	438	98	302	400	109
Average	478.8	104.2	356.8	455.0	105.6
	B. Methyl linolate ½ drop daily				
137	478	105	370	475	100
144	501	73	456	529	95
149	408	111	423	535	76
Average	462.3	96.3	416.3	513	90.3
	C. Methyl linolate 1 drop daily				
150	314	75	263	338	93
122	311	83	252	334	93
142	552	85	429	514	107
132	369	87	300	387	95
Average	386.5	82.5	311.0	393.25	97.0

was used for the titration. The total lipids represent the sum of the cholesterol and total fatty acids. The iodine number of the total lipids was obtained by dividing the milligrams iodine absorbed per unit of serum by the milligrams of total lipid per unit. Duplicate determinations were made on the specimens from the larger rats and on the pooled samples. The results of the experiment are presented in tables 1 to 3.

DISCUSSION

The values for the cholesterol and total fatty acids were essentially the same, both in the rats which had been fed on the highly purified, fat-free diet and in the control animals reared on the stock diet. The total iodine absorbed per unit of serum, however, was lower in the animals of the fat-free group. The iodine number of the total lipids was definitely lower in the group which had been reared on the fat deficient diet (tables 1 and 2). In that cholesterol has an iodine value of 66, it is possible to calculate the iodine value of the total fatty acids of the serum. By this means the average iodine number of the serum fatty acids of the fat-free animals was found to be 103 as compared with 135 for control animals on stock diet. In the larger samples of blood obtained by pooling the serum of several animals, it was found that the iodine number of the total lipids for the fat-free animals was definitely low (table 2). The iodine number of the total fatty acids tends to be lower in the younger age group in human beings (Hansen, '37). A similar finding was obtained in the group of four animals 2 months of age (table 1). The iodine numbers of the total lipids were definitely higher in the group of animals (table 1) which had been given the stock diet only in quantities sufficient to allow them to grow at a rate comparable to the average of that of the fat-free group. The serum cholesterol values in this group were found to be unchanged but the total fatty acids were definitely low. The authors in studying the effect of a high-fat diet in rats observed that the level of the fatty acids of serum tends to vary inversely with their degree of unsaturation. The significance of this is not definite since the character of the body lipids has not as yet been determined. However, it suggests the possibility that the more saturated fats have been utilized during the period of partial starvation.

Esters of linolic acid produce a striking improvement in the condition of the rats suffering from the unsaturated fatty acid deficiency disease. Even when given in small amounts, the animals resume their normal rate of growth, the hematuria disappears and the skin condition clears up. A group of

animals was placed upon various small quantities of the methyl esters of linolic acid and the serum lipids were determined. The findings of this study are presented in table 3. The group of animals which had been given 1 drop daily of the linolic acid ester continuously with their fat-free diet were healthy appearing animals, but the average iodine number of blood lipids was only slightly raised. The rats which had been given only $\frac{1}{2}$ drop of methyl linolate were found to have a symptomatic cure, but the blood lipid iodine numbers were essentially the same as those of the fat-free group. These observations indicate that when the esters of linolic acid are given in minute quantities to rats on the fat-free ration, although they produce a symptomatic cure, they cause only a slight increase in the iodine value of the serum lipids.

The esters of oleic acid were fed in larger amounts than were the esters of linolic acid. There was a slight clinical improvement in these rats although they were by no means normal, healthy animals. Esters of oleic acid are difficult to prepare in pure form, linolic acid esters being the most likely contaminant. Traces of linolic esters present in the methyl oleate preparations may have been responsible for the improvement that occurred in these animals (Burr, Burr and Miller, '32). The iodine values of the serum lipids were somewhat higher than those of the animals given the linolic ester supplements (table 3). The fact that the esters of oleic acid were administered in larger amounts than were the esters of linolic acid no doubt accounts for the greater degree of unsaturation of the serum lipids in this group.

SUMMARY AND CONCLUSIONS

1. The serum lipids of rats reared on a fat-free diet have a lower degree of unsaturation than do the serum lipids of rats on stock diet.

2. Young animals tend to have a lower iodine number of the serum lipids than do normal adult animals on the same diet.

3. When the food intake of the animals on stock diet was so restricted as to hold their weight to the level of the average of the fat-free group, it was found that the degree of unsaturation of the total lipids of the serum was greater than in the normal animals on the same diet unrestricted.

4. Animals given small quantities of the methyl esters of linolic acid sufficient to effect a clinical cure of the unsaturated fatty acid deficiency disease were found to have a slight but definite increase in the iodine number of the total lipids.

5. Esters of oleic acid given in fairly large quantities to animals on the fat deficient diet were able to cause a definite increase in the iodine values of the total lipids, even though they effected only a partial clinical cure.

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REVIEW

RECENT STUDIES OF VITAMINS REQUIRED BY CHICKS *

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The nutritional requirements of chicks have long been known to be incompletely supplied by purified diets which are suitable for rats. Recent investigations indicate that some of the missing factors are being found. For example, within the past 2 years the antihemorrhagic vitamin, essential for the chick, but not found necessary in rat diets, has been purified to a high degree. Comparative studies with rats and chicks have paved the way for the discovery of the different forms of vitamin D. Such comparative studies promise to be of assistance in unravelling the complexities of the water-soluble group of vitamins.

Many papers which deal with poultry nutrition are published annually, and it is beyond the scope of this review to deal with more than a small proportion of such reports. An attempt has been made to confine the review a) to investigations dealing with new dietary essentials, usually of a vitamin-like nature, and b) to recent reports of quantitative experiments with the older and better-known vitamins.

* Exigencies of space have made it necessary to shorten the Literature Cited list of this review. The references indicated by superior numerals may be found in the bibliographies of the following articles:

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PART A. NEW FACTORS

1. The antihemorrhagic vitamin ('vitamin K')

A tendency toward delayed blood clotting in chicks fed on certain simplified diets was observed by Dam ('29) and by McFarlane and co-workers ('31). The latter investigators noted that the condition was characteristic of the feeding of protein supplements which had been extracted with ether. Holst and Halbrook ('33)[6] also described the condition, and found that a cure was brought about by feeding small quantities of cabbage. Dam and Schonheyder ('34)[4] produced the dietary hemorrhagic disease in chicks, and showed that the disease was not caused by a deficiency of any of the known vitamins. Dam ('35) and Almquist and Stokstad ('35),[2] working independently, noted that the protective factor was fat-soluble and unsaponifiable. Dam found the factor to be present in hog liver fat, hempseed, tomatoes and kale. He suggested that it be named 'vitamin K' (Koagulations vitamin). Almquist and Stokstad found that an ether extract of dehydrated alfalfa was an excellent source of the factor, and that the factor was synthesized in fish meal when it was allowed to putrefy slightly, potent extracts of such fish meal being obtained. The vitamin did not promote growth. Later work by Almquist ('36 a) reported concentration of the vitamin from a hexane extract of alfalfa meal. A reddish oil was obtained which was treated with digitonin to remove a small quantity of sterols. The oil was bleached to a light yellow color by treatment with activated magnesium oxide. It afforded complete protection against hemorrhages when fed at a level of 2 parts per million of diet. The factor appeared to be stable to heat and light.

Dam and Schonheyder ('36) also prepared concentrates of the vitamin from extracts of dried alfalfa. They removed inactive material from a light petroleum solution by shaking with 90% methanol. The vitamin was then adsorbed on sugar, which brought about a fivefold concentration. They confirmed Almquist's ('36 a) report that the vitamin was rapidly destroyed by alcoholic potash.

Schonheyder ('36) proposes for vitamin K a unit which is, in substance, that amount of the vitamin which is required per gram of a deficient chick to be orally administered on 3 successive days in order to render normal the clotting power of the blood. A deficient chick weighing 333 gm. would therefore require 1000 units. Almquist's ('36 a) quantitative estimations are based on the level which must be fed in the basal diet to protect chicks against hemorrhages. It is difficult to compare the potency of Almquist's concentrate with Dam and Schonheyder's material, but, on the basis of feed intake, it appears that Almquist's concentrate has a potency of about 10^7 Schonheyder units per gram, and is therefore the most potent concentrate yet reported. More recently, Almquist ('36 b) has reported a several-fold increase in potency produced by distillation of his concentrate in a molecular still.

Some physical and chemical properties of highly potent concentrates of the vitamin, purified by distillation in vacuo were described by Almquist ('37). Molecular weight determination by the camphor method gave a value of about 600. The concentrate contained a trace of nitrogen; phosphorus and sulphur were absent. The light yellow color of the concentrate was attributed to carotene, the presence of which was demonstrated. Other evidence indicated that the antihemorrhagic vitamin is colorless. It was not affected by treatment with phenyl isocyanate or di-nitro benzoyl chloride, reagents for the hydroxyl group, or by di-nitro phenyl hydrazine. Its potency was destroyed by cold bromination and by perbenzoic acid, indicating the presence of unsaturated linkages, and by direct sunlight. The vitamin appeared to be a very complex substance primarily hydrocarbon in structure.

Almquist and Stokstad ('36 b) described conditions influencing the incidence of the hemorrhagic syndrome. The vitamin was found in chick droppings collected in a bactericidal solution, although the chicks were on a diet deficient in the vitamin. This showed bacterial synthesis of the factor in the intestine. Transfer of the vitamin to the chick from the diet of the hen was shown to take place via the egg yolk. The livers

of young chicks on normal diets contained no appreciable quantity of the antihemorrhagic vitamin.

Mode of action of the antihemorrhagic vitamin. The current explanations of normal blood clotting as given in an ordinary physiological textbook consist merely of applying names and ascribing functions to certain crude blood and tissue fractions. Consequently the mode of action of 'vitamin K' is an unsolved physiological problem of the greatest interest. Schonheyder ('36) and Dam, Schonheyder and Tage-Hansen ('36) have studied the question, and reported that the prothrombin fraction from normal chick plasma brought about clotting of deficient plasma in vitro. Concentrates of 'vitamin K' itself were not active in vitro, but the vitamin was present in the prothrombin fraction of normal plasma. It could not be removed from this fraction by washing with acetone and ether, although the vitamin in the free state is soluble in these solvents.

2. Gizzard erosions of dietary origin in chicks

It was reported by McFarlane and co-workers ('31)[2] that the lining membranes of the gizzards of chicks fed on certain simplified diets were eroded and exfoliated. Gizzard erosions in chicks were also reported by Ringrose, Norris and Heuser ('31),[1] in their description of a pellagra-like syndrome in chicks, and by Holst and Halbrook ('33),[6] Dam and Schonheyder ('34)[4] and Almquist and Stokstad ('35)[2] in their respective accounts of the hemorrhagic syndrome. When the basal diets described in these five reports are compared, it is interesting to note that all of them were free from leafy materials, such as alfalfa meal, which are commonly found in practical chick rations. However, Jungherr ('36) reported the condition in chickens given the usual practical rations.

Steps toward the preparation of a dietary factor preventing gizzard erosions were described by Almquist and Stokstad ('36 a).[2] The saponifiable fraction of kale lipids was found to be a potent source of the factor, while copious amounts of the known vitamins, including the antihemorrhagic vitamin,

were without effect. Kline and co-workers ('36),[4] working with an entirely different basal diet, reported that dried lung tissue protected against gizzard erosions, and that the potency was not extracted from lung tissue with hot water. They state that lack of the substance preventing occurrence of lesions in the gizzard had a profound effect upon growth of chicks, due to 'poor absorption.' Almquist and Stokstad ('35),[2] however, found gizzard erosions to occur in rapidly growing birds, and Jukes ('36)[2] stated that growth was not affected by gizzard erosions. The fact that the gizzard is not essential to the absorption of food has been clearly shown by Fritz, Burrows and Titus ('36), who found excellent health to persist in gizzardectomized birds.

Almquist and Stokstad ('37), in a recent publication, report that the factor preventing gizzard erosions is fat soluble. They found it to be easily destroyed by heat and to some extent by alcoholic potassium hydroxide. It was readily adsorbed from solution in hexane by activated magnesium oxide. The best practical sources of the factor were fresh or dried green leaves, and wheat bran. Chick diets containing adequate amounts of wheat bran and dried alfalfa produced very slight, if any, gizzard erosions.

In a recent article, Bird, Kline and co-workers ('36), state that the anti-gizzard-erosion factor was insoluble in ether and in ethyl alcohol, but followed the alkali-soluble acid-precipitable proteins in the fractionation of lung tissue, which was one of the best sources of the factor. Pork liver and kidney were also excellent sources, and oats, wheat bran and middlings were superior to wheat and corn. The factor was quite thermostable in lung tissue, but was destroyed readily in grains either by dry heat or autoclaving. From these results one is led to the conclusion that the factor described by the Wisconsin group must be different from the factor of Almquist and Stokstad ('36 a),[2] but comparison of results on widely differing basal rations is extremely difficult.

3. Nutritional encephalomalacia

It was observed by Drummond ('16)[6] that chicks could not live on a 'simplified diet.' Many unsuccessful attempts have since been made to devise 'purified diets' for chicks. Failure usually results when a purer source of carbohydrate such as starch, dextrin or sucrose is used in place of grain in a chick diet. Recently Hogan, Boucher and Kempster ('35) have been able to raise chicks on 'simplified' rations, which contained such materials as casein, cornstarch, cellophane, lard, wheat germ oil, acid hydrolyzed yeast, liver extract, tikitiki, salt mixture, carotene and irradiated ergosterol.

One of the earlier complications usually observed in chicks on purified diets is characterized by acute paralytic symptoms which simulate polyneuritis. The symptoms and pathology of this nutritional disorder ('encephalomalacia') which is quite distinct from vitamin B deficiency, were first described by Pappenheimer and Goettsch ('31).[6] The most characteristic macroscopic post-mortem symptom is the existence of discolored spots on the surface of the cerebellum, and edema of the brain.

The discovery of a preventive factor in vegetable oils was announced by Pappenheimer and Goettsch ('34). A simultaneous report by Adamstone ('34) stated that the symptoms could be produced by feeding a diet which had been subjected to the Waddell and Steenbock ('28) treatment with ferric chloride for the destruction of vitamin E. Goettsch and Pappenheimer ('36) stated that the non-saponifiable fraction of soy bean oil was a good source of the anti-encephalomalacic factor and that fractions could be prepared which would protect when fed at a level of 0.024%. The factor was stable to autoclaving. Saponification of soy bean oil destroyed the factor unless air was excluded. In the original vegetable oil, however, it was resistant to aeration. Ninety-five per cent alcohol extracted much of the factor from vegetable oil. Corn, cotton seed, peanut and soy bean oils all contained the factor. In contrast to the findings of Adamstone ('34), nutritional encephalomalacia was not produced in chicks by feeding a natural diet

in which vitamin E had been completely destroyed by ferric chloride treatment. Addition of certain foodstuffs rich in vitamin E did not protect chicks on the basal simplified diet, which consisted of starch, dried skim milk, commercial casein, lard, bakers' yeast, paper pulp, salt mixture and cod liver oil.

Vitamin B_4. A water-soluble factor, designated 'vitamin B_4,' was stated by Keenan, Kline, Elvehjem, Hart and Halpin ('33)[4] to be necessary to prevent paralysis in chicks. The paralysis developed on a diet of casein, dextrin, salt mixture, yeast and cod liver oil, and was accompanied by a degeneration of the brain similar to that described by Pappenheimer and Goettsch ('31).[6] A later communication by Kline, Bird, Elvehjem and Hart ('36)[4] describes an improved basal ration for the study of nutritional paralysis in chicks consisting of dextrin, purified casein, salt mixture, brewers' yeast, autoclaved liver residue, crude liver extract powder, water-extracted lung tissue and cod liver oil. The addition of 15% of peanuts, brain or kidney produced normal chicks and fair success was reported with 'certain B_4 concentrates' (undescribed). It is not yet possible to suggest any relationship between the syndrome described by Pappenheimer and Goettsch and that observed by the Wisconsin workers, and characterized by similar symptoms. Goettsch and Pappenheimer ('36) point out that the fact that their preventive factor was present in the non-saponifiable fraction of soy bean oil excludes the possibility that it was 'vitamin B_4' or any other water-soluble vitamin.

Once again, the differences in the basal diets used by different laboratories make comparison of the results difficult. If the two factors are different, it should be possible, to devise a diet which is deficient in both of them, and which will permit normal development only when supplemented with both the vitamin of Pappenheimer and Goettsch and with the 'vitamin B_4' of the Wisconsin workers. In the meantime, it is worthy of note that Kline, Bird, Elvehjem and Hart ('36) reported that hydrogenated cottonseed oil[7] was protective against 'vitamin B_4' deficiency and Arnold, Kline,

[7] Crisco.

Elvehjem and Hart ('36) fed peanut oil 'to supply vitamin B_4.' Goettsch and Pappenheimer ('36) had previously observed that these oils contained their fat-soluble anti-encephalomalacic factor.

4. Vitamin G and associated factors

The need of the chick for a thermo-stable member of the vitamin B complex was first demonstrated by Hauge and Carrick ('25).[1] A pellagra-like syndrome in chicks was produced by Ringrose, Norris and Heuser ('31),[1] who showed that the symptoms could be prevented by including autoclaved yeast or a 'milk vitamin concentrate' in the diet. Later work by Norris and associates (Norris, Heuser, Ringrose, Wilgus and Heiman, '34;[1] Wilgus, Norris and Heuser, '35 a;[1] Davis and Norris, '36) and by Bethke and co-workers (Bethke, Record and Kennard, '33, '36;[1] Bethke, Record and Wilder, '36 a) demonstrated the great importance of 'vitamin G' in poultry feeding.

It was found by Kline, Keenan, Elvehjem and Hart ('32, '33)[2] that the pellagra-like syndrome discovered by Ringrose, Norris and Heuser ('31)[1] could be greatly intensified by feeding chicks a diet which had been subjected to dry heat treatment. The diet consisted of yellow corn meal, wheat middlings and commercial casein heated in an air oven at 100° for 144 hours or 120° for 24 hours (Keenan, Kline, Elvehjem and Hart, '35)[4] and supplemented with minerals and cod liver oil. This discovery by the Wisconsin workers led to rapid advance in the study of the vitamin G group of factors with chicks. Elvehjem and Koehn ('35)[1] found that the syndrome produced by the heated diet was cured by the filtrate from an aqueous extract of liver after flavin had been removed by adsorption on fuller's earth. The addition of flavin to the basal diet intensified the symptoms. It was proposed to apply the name 'vitamin B_2' to the factor in the filtrate rather than, as Kuhn and others have, to flavin. The results of Elvehjem and Koehn were confirmed by Lepkovsky and Jukes ('35),[1] who noted that basal diets similar to that employed

by Elvehjem and Koehn, or by Ringrose, Norris and Heuser, tended to be deficient in flavin, and that flavin had a marked growth promoting action on the chick when fed with a diet containing an adequate supply of other vitamins, including the factor in the filtrate.

Thus two factors in the vitamin G group have been shown to be essential for chicks. Further work will be needed to establish the exact role of each of these factors in poultry nutrition, since many experiments on 'vitamin G' in poultry feeding undoubtedly dealt with a mixture of the two factors, both of which are strongly growth promoting. The factors are: 1) flavin, 2) the factor curing the dermatitis produced by the heated diet, called 'vitamin B_2' by Elvehjem and coworkers; and the 'filtrate-factor' by Lepkovsky and Jukes. It is not advisable to apply the term 'chick anti-dermatitis factor' to the filtrate factor, since Lease and Parsons ('34) showed that there are two 'chick anti-dermatitis factors.'

The nomenclature of vitamin G(B_2) is in a confusing state. Elvehjem and Koehn apply the term 'vitamin B_2' to the factor curing the heated diet syndrome in chicks. Strong support is lent to this terminology by their recent experiments (Koehn and Elvehjem, '36)[2] which showed that concentrates of the factor would cure blacktongue in dogs, regarded as the analogue of pellagra in man. Many workers, on the other hand, refer to lactoflavin as 'vitamin G' on the basis of the large number of 'vitamin G' studies which have been carried out in recent years with chicks and rats in which lactoflavin was probably the primary factor involved. Yet another attitude is adopted by György ('35)[1] and others who speak of a group of factors as the 'vitamin B_2 complex,' and undoubtedly the 'vitamin G(B_2)' assays of recent years were measurements of a mixture of factors. These differences of terminology could probably be easily abolished by a committee on nomenclature.

Lepkovsky and Jukes ('36)[3] found that chicks on a diet low in flavin but high in the filtrate factor grew very slowly and developed diarrhea. No dermatitis was observed, and growth of feathers seemed unimpaired. The symptoms were

prevented and growth was restored by the addition of crystalline lactoflavin or hepatoflavin to the diet. The great importance of flavin is also indicated in reports by Bethke, Record and Wilder ('36 b) and Davis, Norris and Heuser ('36). The first-named workers found that pure flavin increased growth of chicks and prevented the occurrence of a leg disorder (a flexion of the toes with medial rotation of the hock) first described by Norris, Heuser and Wilgus ('29, '30).[1] Davis, Norris and Heuser stated that feeding 600γ of 'purified extract of vitamin G' per hen per week restored normal hatchability to eggs produced on a basal ration deficient in 'vitamin G.'

Findings at Cornell were summarized and analyzed in a recent bulletin by Norris, Wilgus, Ringrose, Heiman and Heuser ('36).[1] A chick unit of 'vitamin G' approximately equal to 1 microgram of flavin was proposed, and many feeding stuffs were quantitatively assayed for 'vitamin G.' It was found that chicks needed approximately 290 units of 'vitamin G' per 100 gm. of feed in order to attain normal weight at 8 weeks of age. Hens required about 230 units per 100 gm. of feed in a breeding diet, but 130 units sufficed for normal egg production. In the writer's experience, diets such as the chick basal diet used for vitamin G assay are deficient not only in flavin, but also in the strongly growth-promoting filtrate factor. The vitamin G standard was dried pork liver, which is rich in both factors. Hence the 'vitamin G' unit was probably based, not on flavin alone, but on a composite growth-promoting effect of flavin and the filtrate factor.

The properties of the filtrate factor ('vitamin B_2' of Elvehjem) were studied by Elvehjem and Koehn ('35)[1] and by Lepkovsky and Jukes ('36).[2] It was reported to be colorless, soluble in water and moist, weakly polar solvents; but insoluble in ether. It was found not to be adsorbed on fullers' earth, charcoal or lead sulfide. Exposure to visible light did not destroy the factor. It resisted treatment with several oxidizing and reducing agents. In a later report (Jukes, '37)[2] the factor was stated to be soluble in 99.5% ethanol and in 25%

phosphotungstic acid and was not destroyed by benzoyl chloride.

Jukes and Lepkovsky ('36)[2] described a method for quantitative assay of the factor, using a heated diet to which had been added a fuller's earth adsorbate of whey to supply lactoflavin, and reported the distribution of the factor in some feeding stuffs. Growth-promotion by the factor was found to increase in linear proportion to the amount of the factor which was fed, until a maximal growth was reached. The daily dose of the factor which just sufficed to produce this maximal growth in a 3-week-old chick was defined (Jukes, '37)[2] as 10 units. Since the food intake of such a chick is about 10 gm., the requirement for growth is about 100 units per 100 gm. of feed. Cane molasses appeared to be the cheapest concentrated source. Further assays (Jukes, '37)[2] indicated marked differences between the distribution of the filtrate factor and the human P-P factor in natural foods. Egg yolk was quite rich in the filtrate factor.

Curiously enough, lactoflavin, while not preventing or curing dermatitis in chicks, appears to be the anti-dermatitis vitamin of the turkey (Lepkovsky and Jukes, '36).[2]

No results have yet been reported on the effect on chicks of a deficiency of 'vitamin B_6' (György, '35 b)[1] preventing an acute dermatitis in rats ('factor 1' of Lepkovsky, Jukes and Krause, '36).[2] 'Vitamin B_6' is quite distinct from the 'filtrate factor.' The differences are summarized in table 1.

5. Other factors

Kline, Elvehjem, Keenan and Hart ('34)[4] reported a growth factor for chicks present in water-extracted liver residue. The factor was very stable to heat, since it resisted autoclaving at its natural pH for 10 hours, and was not destroyed by dry heating at 120° for 6 days. It was destroyed in liver residue by autoclaving at pH 9 for 5 hours at 15 pounds pressure. It was insoluble in water until the liver had been mildly hydrolyzed with acid. After this it could be partially extracted with normal butyl alcohol. The factor was later

found to be arginine (Arnold, Kline, Elvehjem and Hart, '36). This observation is very interesting in view of the fact that the basal diet was apparently well supplied with arginine, and addition of casein was ineffective.

Nestler, Byerly, Ellis and Titus ('36) fed hens a basal feed mixture containing "at least enough vitamin G to meet the minimum requirements of chickens for hatchability." The

TABLE 1

Differences between 'vitamin B_6' and the 'filtrate factor' (chick anti-dermatitis vitamin)

'Vitamin B_6' rat antidermatitis factor	*'Filtrate factor,' chick anti-dermatitis factor, 'vitamin B_2' of Elvehjem and Koehn ('35)*
Cures characteristic dermatitis in rats (by definition)	Does not cure rat dermatitis [a]
Does not cure chick dermatitis produced by heated diet [a]	Cures chick dermatitis produced by heated diet (by definition)
Adsorbed by fuller's earth in acid solution [a, b]	Not adsorbed by fuller's earth [a, c]
Destroyed by benzoyl chloride [b]	Resistant to benzoyl chloride [d]
Destroyed by visible light [e]	Not destroyed by visible light [e]
Extracted from feeding stuffs by water with much difficulty [b]	Readily extracted from feeding stuffs by water [d]
Precipitated by phosphotungstic acid [b]	Not precipitated by phosphotungstic acid [d]
Marked differences in natural distribution [d, f, g]	

[a] Lepkovsky, Jukes and Krause ('36).[2]
[b] Birch and György ('36).[3]
[c] Elvehjem and Koehn ('35).[1]
[d] Jukes ('37).[3]
[e] György ('35).
[f] Birch, György and Harris ('35).
[g] Jukes and Lepkovsky ('36).[3]

ration lacked a factor necessary for high hatchability which was relatively abundant in dried pork liver meal. Hatchability was also improved by allowing the birds to range on grass and some improvement was effected by addition of a mixture of meat meal, fish meal and buttermilk. Dried whey produced little improvement. The nature of the factor, and its possible relation to other dietary essentials, including amino-acids, is not clear from the results.

The egg white syndrome. The work of Lease and Parsons ('34) showed that the symptoms produced in chicks by adding fermented egg white to a complete diet were cured, not by liver extract, but by liver residue. The symptoms simulated the dermatitis produced by feeding a heated diet of natural foodstuffs. The heated diet dermatitis, however, was cured, not by liver residue, but by liver extract. Heiman ('35)[1] found that dried skim milk or dried whey, at levels as high as 20%, did not alter the severity of the pellagra-like syndrome produced by feeding a diet containing dried egg white. Dried skim milk and dried whey are fairly good sources of the 'filtrate factor' (Jukes and Lepkovsky, '36)[2] curing the heated diet dermatitis. Hence the egg white syndrome appears to have an entirely different etiology from the heated diet dermatitis in spite of the similarity in appearance of the two conditions.

6. Slipped tendon ('perosis') and manganese

The hock deformity known as 'slipped tendon' or 'perosis' is associated with high levels of phosphorus in the diet of growing chicks (Milby, '33).[6] Hogan, Shrewsbury and Kempster ('28) found that the bones in chicks with slipped tendons were of normal ash content and that the percentages of calcium and inorganic phosphorus in the blood were normal, thus sharply differentiating the condition from rickets. It was noted by Card ('29, '30) that excessive quantities of bone meal in the ration tended to cause slipped tendons.

The discovery of a separate preventive factor was first announced by Lee ('29), who reported that a 'leg weakness,' not prevented by cod liver oil or yeast, was largely prevented by the addition of 15% of either bran or middlings to the ration in addition to the use of cod liver oil. Oats or oat feed were reported by Hunter, Dutcher and Knandel ('30–'31),[6] and rice bran by Titus ('30–'31), to possess beneficial properties for the prevention of slipped tendons. Titus ('32)[6] suggested that the preventive factor possibly belonged to the vitamin B complex. Sherwood and Couch ('33) reported

protective results with rice bran and oat groats, and found that the preventive factor was also present in wheat gray shorts. Wilgus, Norris and Heuser ('35) determined the perosis-preventing properties of certain feed stuffs, and found the relative effectiveness to be as follows: Wheat germ, 100; wheat standard middlings, 65; red dog flour, 50; wheat bran, 40; ground oats, 30; hard wheat, 5; and soy bean oil meal, 0. The effectiveness of ground yellow corn and alfalfa meal could not be determined in this manner, but, by indirect means, both of them were found to have a slight preventive action. It was found by Serfontein and Payne ('34) that a tendency toward perosis could be inherited. The sum of evidence made it apparent that several conditions contributed to the etiology of perosis.

Recently Wilgus, Norris and Heuser ('36) have announced that either 0.0035 or 0.0160% of manganese in the diet is quite effective in preventing perosis at levels of calcium of 1.0 and 1.2% and of phosphorus of 0.8 and 1.2%. Aluminum and zinc possessed some preventive action. The perosis preventing property of feed stuffs was roughly related to their manganese content. Almost simultaneously, Sherwood and Fraps ('36) reported that the protective factor in wheat gray shorts is one or more of the mineral constituents of the ash.

PART B. RECENT STUDIES WITH VITAMINS A, B(B_1), D AND E

1. Vitamin A

The importance of vitamin A in poultry nutrition is very great. The subject was ably reviewed by Cruickshank ('35–'36).[6] Cruickshank's review included a description of the pathological symptoms of vitamin A deficiency in chickens. Our knowledge of the pathology has been since supplemented by Hinshaw and Lloyd ('34)[5] who made a comparative study of vitamin A deficiency in chicks and turkeys.

Recent work, which will be dealt with in this review, deals principally with quantitative aspects of the vitamin A requirement of chickens.

a. Vitamin A requirements of the growing chick. The chick is hatched with a reserve of vitamin A in the yolk sac and the liver, and estimations of the vitamin A requirement of growing chicks are hence often based on studies of birds at 8 weeks of age, thus allowing time for the reserves to become exhausted.

Frohring and Wyeno ('34)[5] found, by a rather involved calculation, that the minimum vitamin A requirement of an 8-week-old chick was about 92 international units per day when fed as a daily dose of carotene in cottonseed oil. A similar result was obtained with cod liver oil. Record, Bethke and Wilder ('35)[5] reported that 50 to 100 micrograms of carotene (80 to 170 international units as beta-carotene) per 100 gm. of feed were required for normal growth to 8 weeks of age. The same amount, in terms of rat units, was required when the vitamin was supplied as cod liver oil. Wilson, Schroeder and Higgins ('36) also reported that, unit for unit, carotene and vitamin A obtained from a fish oil concentrate were utilized equally well by the chick. Ringrose and Norris ('36)[5] used carotene as a standard in a careful study of the vitamin A requirements of the growing chick. They found that 100 international units per 100 gm. of feed were just sufficient for prevention of symptoms up to 8 weeks of age but not sufficient to allow normal growth. One hundred and fifty units was practically as satisfactory for growth as higher levels. Yellow corn was found to furnish about 7 units of vitamin A per gram. Smith ('33)[5] found that between 12.5 and 25% of yellow corn supplied the vitamin A requirement. This would correspond to between 88 and 175 international units of vitamin A per 100 gm. of feed if the corn contained 7 units per gram. Wilson, Schroeder and Higgins ('36) stated that 264 international units per 100 gm. of feed were adequate to promote satisfactory growth. The minimum requirements were not stated.

Biely and Chalmers ('36 a) fed vitamin A to chicks by pipette in weekly doses. By means of this technic, they found that the weekly administration of 525 units of vitamin A was

sufficient to ensure normal growth and protection against deficiency symptoms up to 8 weeks of age. The method is not easily comparable with normal feeding practice, since the dose did not vary with the steadily increasing food intake of the growing chick. Hence a chick might tend to store vitamin A at the beginning of the experiment, and protect itself from deficiency in the later stages by drawing on its store. The same authors ('36 b) reported that there were indications that sardine oil (pilchard oil) contained some substance which chicks, but not rats, could utilize as a source of vitamin A, but a contrary opinion was expressed by Milne ('36), who found no indications that pilchard oil (sardine oil) contained any growth promoting factor which was not vitamin A.

b. Vitamin A requirements of laying hens. The vitamin A requirements for egg production have been extensively studied by Sherwood and Fraps ('33, '34, '35).[5, 6] They express their results in Sherman-Munsell units, which have been converted to international units in this review. In their first report it was noted that a mash containing 20% of ground yellow corn, supplemented with yellow corn as scratch grain, failed to supply the requirements, since the vitamin A content of the yolk decreased from 28 units to from 7 to 11 units during the experiment. It was calculated that 6.3 units in the feed were required to produce 1 unit in the egg, and the maintenance requirement was estimated at 150 units per day. The use of dried alfalfa was stated to be insufficient to supply the requirements, and green feed was recommended in addition. In 1934 the same authors noted a decline in the vitamin A content of the egg yolk during the laying season even when the hens received 630 units per day. The hens receiving 630 units, supplied as dried alfalfa, laid about 15% more eggs than hens receiving 320 and 480 units. However, it appears to the writer that the rations supplying the lower levels were probably deficient in lactoflavin. Four units of vitamin A in the feed were required to produce 1 unit in the egg. Green feed was again recommended as being necessary to supplement the ration. However, it seems that the alfalfa meal

used was of distinctly low potency (110 units per gram). Sherwood and Fraps ('35) continued the investigation and found that the vitamin A requirements for the formation of feathers seemed to be as high as the requirements for egg production, since molting hens did not store the vitamin even when fed liberal amounts. The authors estimated that 340 units per day, or about 430 units per 100 gm. of feed were required to keep the hens in good health and maintain a good production of eggs. Once again, it appears that the ration was somewhat low in lactoflavin.

Russell and co-workers ('36) found that increasing the vitamin A content of a ration which contained 485 units per 100 gm. did not improve egg production and hatchability and did not reduce mortality. It was concluded that the usual type of laying ration, containing yellow corn, alfalfa and fish liver oil, supplied sufficient vitamin A.

2. *Vitamin B(B_1)*

Chickens were the first animals to be used in experimental vitamin B studies. In spite of this, very little is known about the quantitative vitamin B requirements of the chick. This lack of information may be traced to two causes; first, it is difficult to devise a vitamin B-free ration for chicks which supplies all other essentials, second, lack of interest has been caused by the fact that practical poultry rations apparently more than sufficiently meet the vitamin B requirements of the chick.

Kline, Keenan, Elvehjem and Hart ('32–'33)[2] found that chicks developed acute symptoms of polyneuritis when fed an autoclaved ration consisting of yellow corn, 58%; wheat middlings, 25%; casein, 12%; minerals and cod liver oil, 5%. When 16% of the unheated ration was added to the autoclaved ration, some protection was afforded, and when 32% was added, the chicks grew 'normally.' Hence it appears that the unheated ration, if fed alone, would have supplied at least three times the necessary amount of vitamin B(B_1) for chicks.

3. Vitamin D

The discovery 15 years ago of the need of poultry for the antirachitic factor has had a profound influence on feeding practice. Space does not permit a discussion of more than a few of the recent investigations of vitamin D which have been made with chicks.

a. The forms of vitamin D and their varying effectiveness for chicks and rats. Following the original discovery of Carrick (Bills, '35),[3] it was clearly shown by Massengale and Nussmeier ('30),[3] Mussehl and Ackerson ('29, '30)[6] and Hess and Supplee ('29, '30)[6] that cod liver oil, rat unit for rat unit, is superior to irradiated ergosterol as a source of vitamin D for chicks. Subsequent investigators have repeatedly confirmed this finding. The difference is not due to poorer uptake of irradiated ergosterol, because Klein and Russell ('31)[6] found that of the total amount of vitamin D fed as irradiated ergosterol to chicks during the first 4 weeks of life, 26.5% of the rat units consumed were recovered in the droppings, and when cod liver oil was fed instead, the recovery was 43.1%. Russell, Taylor and Wilcox ('32–'33) also noted that essentially the same bone ash percentages were obtained by administering vitamin D to chicks by injection as when it was fed by capsule. Irradiated ergosterol and cod liver oil were both used. The relative superiority of 1 rat unit of cod liver oil to 1 rat unit of irradiated ergosterol for the chick has been found to range from fifteen- to twentyfold (Bethke, Record and Kennard, '33)[3] to 144- to 192-fold (Russell, Taylor and Wilcox, '32–'33). Waddell ('34) clearly demonstrated that irradiated cholesterol is much more efficacious, rat unit for rat unit, than irradiated ergosterol in preventing rickets in chicks. This finding by Waddell immediately draws attention to the possibility that other sterols were contaminants of certain of the ergosterol preparations used in making comparisons between rats and chicks, i.e., that the difference, e.g., between the results of Bethke, Record and Kennard ('33)[3] and those of Russell, Taylor and Wilcox ('34), alluded to above, may be due to varying amounts of traces of other sterols

with pro-vitamin D activity in the various samples of ergosterol used. Opposition to this suggestion is found in an article by Bills, Massengale, McDonald and Wirick ('35).[3] These workers standardized with rats seven specimens of irradiated partially purified ergosterol, and found the specimens not to differ, one from another, in their effects on the blood and bones of chickens. They found 1 rat unit of vitamin D in cod liver oil to be fifty times as effective for the chick as 1 rat unit of vitamin D in irradiated ergosterol. Previously they had found a difference of 100 times. They comment that this difference is greatly affected by varying experimental conditions.

Bills ('35)[3] has discussed further the standardization of the rat: chick comparison. He draws attention to the importance of the 'response curve' for chicks, obtained by plotting percentage bone ash against vitamin unitage. The 'response curve' has different shapes for different sources of vitamin D, and allowance for this fact must be made in comparing various forms of vitamin D. Presumably it would be of advantage to research workers to use a more standardized chick test for the estimation of vitamin D in order to facilitate the comparison of work carried out by different laboratories. Bills ('35)[3] states further that "unless the test period is extended to longer than 4 weeks one cannot produce quite as hard a bone in chickens with irradiated ergosterol as one can with cod liver oil, no matter how large the dosage given." Massengale and Bills ('36)[3] have also discussed refinements in the chick test.

Certain experiments have indicated that some form or forms of vitamin D are even more effective for chicks, rat unit for rat unit, than the vitamin D of cod liver oil. Bills, Massengale, Imboden and Hall ('36) reported that the vitamin D of white sea bass liver oil was 2.6 times as effective for chicks, rat unit for rat unit, as that of cod liver oil. The latter had the same degree of relative effectiveness as irradiated 7-dehydrocholesterol. Hathaway and Lobb ('36) concluded that the vitamin D of irradiated crude cholesterol was more effective

for chicks, rat unit for rat unit, than that of cod liver oil. Koch and Koch ('36), from spectroscopic studies, inferred that 7-dehydrocholesterol and heated cholesterol were different forms of pro-vitamin D. From comparative experiments with rats and chicks, they concluded that corn phytosterol and ergosterol were two additional forms of the pro-vitamin. Recent conceptions of the structural formulas of certain forms of vitamin D and its precursors were reviewed by Bacharach ('36) and Grab ('36).

A comparison of several irradiated oils was made by Haman and Steenbock ('36). The vitamin D activity of cod liver oil for the chick was found not to be increased by irradiation. Comparative studies showed that the provitamin D of animal fats gave rise to a more effective form of the vitamin, rat unit for rat unit, than the provitamin D of plant oils, when animals fats and plant oils were irradiated and fed to chicks.

The comparative antirachitic efficacy for rats and chicks of several forms of vitamin D was studied by Grab ('36). A tuna liver oil concentrate was used as the standard rather than cod liver oil, and Bills, Massengale, Imboden and Hall ('36) have shown that the efficacy ratio of tuna oils is determined by the species of tuna. Hence it is difficult to compare the results of Grab ('36) directly with those of other investigators. Grab found the efficacy for *rats,* chick unit for chick unit, compared with the efficacy of vitamin D of tuna liver oil concentrate to be for calciferol (vitamin D_2) 32; vitamin D_3, prepared from irradiated 7-dehydrocholesterol 0.65; vitamin D_3, prepared from tuna liver oil 0.60; irradiated 22-dihydroergosterol 2.5 to 10; irradiated 7-dehydrositosterol more than 13. Experiments with 7-dehydrositosterol and 7-dehydrostigmasterol led to the conclusion that 29-carbon-atom sterols were too weak in provitamin D activity to be of any importance either to rats or chicks as sources of the vitamin.

Table 2 summarizes some recent studies on vitamin D. It has not been attempted to list all of the various forms of vitamin D in the table, and certain sources of the vitamin named in the table are probably mixtures, but the tabulated

TABLE 2

Certain sources and precursors of vitamin D and their comparative effectiveness for chicks and rats

SOURCE OF VITAMIN D	PRECURSOR	PROBABLE FORM	EFFECTIVENESS FOR RATS	EFFICACY QUOTIENT, I.E., THE APPROXIMATE EFFECTIVENESS OF 1 RAT UNIT FOR CHICKS AS COMPARED WITH 1 RAT UNIT OF COD LIVER OIL
Calciferol	Ergosterol	Vitamin D_2	40,000 I.U. per mg. [a]	0.02 [b]
Calcium cholesterilene sulfonate	Cholesterol	?	+	>1 [i]
Irradiation product	Unheated crude cholesterol	?	++++	>1 [c]
Irradiation product	Unheated purified cholesterol	?	+	<1 [c]
Irradiation product	Heated purified cholesterol	?	++++	1 [c]
Irradiation product	7-dehydrocholesterol	Vitamin D_3	24,000 I.U. per mg. [e] 25,000 I.U. per mg. [d] 17,000 I.U. per mg. [j]	1 [e]
Tuna liver oil	?	Vitamin D_3	25,000 I.U. per mg. [d] 20,000 I.U. per mg. [j]	See text
Irradiation product	7-dehydrositosterol	?	1/40 activity of irradiated ergosterol [f] 500 I.U. per mg. [j]	See text
Irradiation product	22-dihydroergosterol	?	2000 I.U. per mg. [j] 2500–3100 I.U. per mg. [h]	<1, but more effective than irradiated ergosterol [h]
Irradiation product	Corn phytosterol	?	+	Three times as effective as viosterol [g]
Cod liver oil	?	?	+	1
White sea bass liver oil	?	?	+	2.6 [e]
Albacore liver oil	?	?	+	0.9 [e]

[a] Windaus, Schenck and Werder ('36).
[b] Bills, Massengale, McDonald and Wirick ('35).
[c] Hathaway and Lobb ('36).
[d] Brockmann ('36).
[e] Bills, Massengale, Imboden and Hall ('36).
[f] Wünderlich ('36).
[g] Koch and Koch ('36).
[h] McDonald ('36).
[i] Yoder, Thomas and Lyons ('35).
[j] Grab ('36).

findings are all illustrative of the role of the chick test in the elucidation of vitamin D.

b. The vitamin D requirements of growing chicks. The preceding section has drawn attention to differences in the effectiveness of various forms of vitamin D for the chicks. These differences make it evident that quantitative estimates of the requirements of chicks for vitamin D are dependent

TABLE 3

The vitamin D requirements of growing chicks as determined by means of cod liver oil

INVESTIGATORS	INTERNATIONAL UNITS REQUIRED PER 100 GM. OF FEED	CRITERIA
Bethke, Record and Kennard ('33)	19 [a]	Normal calcification
Russell, Taylor and Wilcox ('34)	15 [b]	Normal bone ash and body weight
Carver, Robertson Braxie, Johnson and St. John ('34)[c]	17	Satisfactory calcification and growth
Murphy, Hunter and Knandel ('36)	19	Bone histology, blood serum calcium, growth
Couch, Fraps and Sherwood ('35)	12.3 for maximum growth 3.1 for prevention of rickets	
Hathaway and Lobb ('36)	27 adequate 13.5 slightly inadequate	Weight gain Bone ash

[a] 7 Steenbock units.
[b] 5.5 Steenbock units.

upon the form of the vitamin used. If we assume that cod liver oil contains only one form of the vitamin or a uniform mixture of two or more forms, it is possible to compare the findings of different workers (table 3). Allowances must be made for genetic differences, various criteria used for estimating rickets, differences in feed consumption, and differences in calcium and phosphorus content of the various rations used.

Among practical sources of vitamin D for chicks, sardine oil (pilchard oil) has been found to be as effective as cod liver oil (Biely and Palmer, '33; Tepper, '35).

c. The vitamin D requirements of laying hens. Carver and co-workers ('34)[6] found that laying hens in confinement without sunlight required 67 international units of vitamin D (cod liver oil) per 100 gm. of feed to secure satisfactory egg production and 'egg quality,' and 135 units to ensure maximum hatchability. Murphy, Hunter and Knandel ('36)[3] found that 78 international (U.S.P.) units of vitamin D (cod liver oil) per 100 gm. of feed for laying pullets, confined without access to sunlight, produced satisfactory results in maintenance of body weight, egg production, egg size, quality of egg shell, and hatchability. Fifty-eight units were insufficient.

d. Ultra-violet irradiation as a source of vitamin D for chicks. The vitamin D requirements of chickens are supplied with remarkable ease by exposure to ultra-violet irradiation. The precursor in the surface of the bird's body has not been identified. The interesting claim has been made by Hou ('29) that the sebaceous secretion of the preen gland is spread by birds over their plumage and then reingested following exposure to sunlight to serve as the principal source of vitamin D for birds. However, Knowles, Hart and Halpin ('35) found that removal of the preen gland from chicks had no depressing effect upon growth or skeletal development when the sole source of vitamin D was irradiation from a quartz mercury vapor lamp.

It was found by Maughan ('28) that the region of the spectrum most effective in curing rickets in chicks was in the neighborhood of 2965Å. Wave lengths of 3130 and longer were ineffective. Wave lengths shorter than 2894 seemed to have little antirachitic power, but this might have been due to the absorption of the shorter rays by the dead cell layer on the surface of the skin. Maughan also noted the great efficiency of the mercury vapor lamp in curing rickets in chicks; an exposure of 1 minute per day at a distance of about 56 cm. brought about 95% recovery in 28 days. Scott, Hart

and Halpin ('29) found that irradiation with a quartz mercury vapor lamp once a week for a 3-minute period at a distance of 56 cm. was sufficient to prevent rickets in chicks.

Light is similarly effective for supplying the vitamin D requirement of laying hens. Carver and co-workers ('34)[6] found that, under the conditions of the experiment, sunshine (sun porches) was sufficient as a sole source of vitamin D to provide for growth of pullets to 24 weeks, and for egg production and hatchability during the summer months. Winter egg production and hatchability were improved by additional vitamin D in the form of cod liver oil. Murphy, Hunter and Knandel ('36)[3] reported that, for the conditions experienced, Leghorn pullets given access to range throughout the entire laying period may secure sufficient vitamin D by irradiation from direct sunshine for satisfactory performance. Body weight, percentage egg production, egg weight, percentage shell of egg, percentage ash of shell, blood serum calcium, and hatchability of fertile eggs were the criteria of performance.

No results have been published which give the comparative efficacy for chicks, rat unit for rat unit, of the vitamin D of cod liver oil and the vitamin D of egg yolk from hens which have received ultra-violet light as the sole source of the antirachitic factor. Such a study might give valuable information on the form of vitamin D which is synthesized with such apparent ease in the skin of the hen.

4. Vitamin E

Ordinary poultry rations contain comparatively large amounts of the germs of grains, and are usually supplemented with fresh or dried greens. Hence it is generally assumed that such rations supply ample amounts of vitamin E. Support for this assumption is found in the results of Card ('28, '29),[6] who observed that the addition of wheat germ oil to a ration containing corn, wheat bran, wheat middlings, meat scrap and salt did not improve fertility or hatchability.

The discovery by Waddell and Steenbock ('28) of the ferric chloride treatment for destruction of vitamin E in natural food stuffs, facilitated studies of the vitamin E requirements of poultry. Card, Mitchell and Hamilton ('30) fed a ration treated in this manner to pullets from 8 weeks of age to maturity. The pullets were mated with males which received vitamin E. Of 354 eggs laid in a period of 53 days, 317 were fertile as shown by candling, but only forty-one developed beyond the ninth day of incubation, and none hatched. Pathological conditions in the embryos in this experiment were described by Adamstone ('31). It is impossible to say whether or not the pathology was influenced by a deficiency of the antihemorrhagic vitamin. The administration of 0.5 cc. of wheat germ oil daily to the pullets brought the hatchability to normal. Discontinuation of wheat germ oil feeding resulted in immediate falling off of hatchability. The results seemed to indicate clearly the necessity of vitamin E in the diet of the hen for hatchability, although egg production and fertility were apparently not greatly affected by vitamin E deficiency. The effects of the same diet on male chickens were studied by Adamstone and Card ('34). After 1 year on the vitamin E-free diet all of the males were capable of fertilizing ova, but after 2 years some of the males were sterile. Histological studies were made and some degenerative changes were revealed. The authors concluded that the testis of the fowl was extremely resistant to vitamin E deficiency.

Barnum ('35) found by means of rat tests that eggs were low in vitamin E if laid by hens on diets designed to be deficient in this vitamin. It was also noted that the eggs were of low hatchability. Hatchability was improved by the addition of 15% of wheat germ to the diet of the hen, but no such improvement was produced by adding lettuce. It has since become apparent from the results of Bethke, Record and Kennard ('36) that wheat germ may contain a factor, not vitamin E, which improves hatchability. The results of Barnum ('35) do not indicate any possibility of a deficiency of vitamin E in the usual poultry rations.

A report by Ender ('35) on improvement of hatchability by wheat germ oil is difficult to interpret because the experiment was not controlled with respect to seasonal variation of hatchability.

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THE IDENTITY OF FLAVIN WITH THE CATARACT-PREVENTIVE FACTOR [1]

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THREE FIGURES

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Evidence from several laboratories indicates that there are at least two heat-stable vitamins of the B complex essential to the nutrition of the rat (Chick, et al., '35; György, '34, '35; Harris, '35; Hogan and Richardson, '36; Bender, et al., '36; Dann, '36; Copping, '36). One of these vitamins, lactoflavin (d-riboflavin), has been isolated, chemically identified, and synthesized (von Euler, '36). The other known factor can at present be identified only by the symptoms that its lack produces in experimental animals. A deficiency of this factor (called vitamin B_6 by many writers) produces in rats a marked inflammation and edema of the paws, ears and nose. The syndrome has been termed 'rat pellagra' by several investigators, 'specific dermatitis' by György ('34, '35), 'florid dermatitis' by Chick, Copping and Edgar ('35), and 'rat acrodynia' by Birch, György and Harris ('35).

A deficiency of flavin produces in young rats a denuded condition, called a 'skin affection' by Chick, Copping and Edgar ('35), and 'non-specific' lesions by György ('34, '35). Since there is usually no inflammation of the skin accompanying this condition, it is not, strictly speaking, a dermatitis. As a loss of hair is the most characteristic feature of the condition, the term 'alopecia' probably describes it as adequately as any single word can do.

[1] Research paper no. 505, journal series, University of Arkansas.

In a series of papers from this laboratory, embracing work extended over a period of more than 6 years, we have reported the consistent appearance of cataract in rats as a result of 'vitamin G' deficiency (Day, et al., '31; Langston and Day, '33, '34; Day, '34, '34 a; Day and Langston, '34; Day and Darby, '36). This has been corroborated by enough widely separated laboratories (O'Brien, '32; Yudkin, '33; Guha, '35; Bourne and Pyke, '35) to render it quite unlikely that the cataract is due to any peculiarity of our experimental technic or strain of animals. Some of these investigators have suggested, however, that cataract may result from a deficiency of some yet unidentified factor.

The availability of pure lactoflavin has made it possible for us to determine whether or not this substance would prevent cataract in our experimental vitamin G-deficient animals. Experiments have been made in which our deficient diet was supplemented with various amounts of pure flavin. The results of these experiments constitute this report.

EXPERIMENTAL METHODS AND RESULTS

The cataract-preventive action of pure lactoflavin. The vitamin G-deficient diet (no. 625) used in the experiment had the following composition: casein, vitamin-free, Labco brand (Suplee et al., '36), 18%; cod liver oil,[2] 2%; butter fat, 8%; salt mixture, Osborne and Mendel ('19), 4%; cornstarch, 68%. The cornstarch carried an extract of rice polish prepared with 80% ethyl alcohol (Day and Langston, '34) evaporated down on the starch and incorporated in the diet so that each 100 gm. of the diet contained the extract of 25 gm. of rice polish. Our method of caging, care and selection of animals has been described (Day and Darby, '36).

Two samples of lactoflavin were used in the feeding experiments. The Winthrop Chemical Company supplied us with some synthetic lactoflavin, and Dr. S. Lepkovsky kindly sent us a small amount of natural lactoflavin that had been prepared in his laboratory. Dr. O. W. Barlow of the Winthrop

[2] Supplied by the E. L. Patch Company, Boston.

Chemical Company has given us the following statement of properties for their synthetic product: melting point, 292°C.—decomposition; melting point of tetra-acetyl derivative, 242°C.; optical rotation in 0.05 *N* NaOH, $[\alpha]_D^{20°} = -110° \pm 5°$; optical rotation in 0.05 *N* NaOH + half saturated borax, $[\alpha]_D^{20°} = 365° \pm 10°$. These properties are in good agreement with the physical constants reported in the literature for

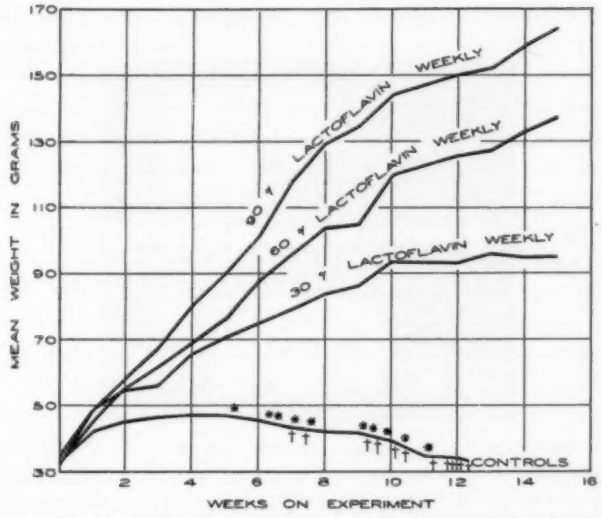

Fig. 1 Composite weight curves of rats receiving the flavin-deficient diet no. 625 (controls), and of rats receiving the same diet supplemented with pure lactoflavin. The asterisks mark the time of appearance of cataract, and daggers mark the death of individual animals. None of the rats receiving flavin exhibited evidence of cataract.

lactoflavin (von Euler, '36). The lactoflavin was fed in weekly doses of 30, 60 and 90 micrograms, to groups of six rats on each level of feeding. Eleven control rats, littermates of the eighteen rats receiving flavin, were given the deficient diet only. Composite weight curves for the eleven control animals and for the rats receiving synthetic flavin at the three levels of feeding are shown in figure 1, and photographs of typical animals are shown in figure 2.

One of the eleven control rats died on the forty-ninth day without exhibiting cataract. The remaining ten rats all showed cataract between the thirty-seventh and seventy-eighth days, as seen with the ophthalmoscope; the average time of appearance was 58 days. Four of the eleven rats exhibited gross, mature cataract, readily evident with the naked eye by the most casual observation. Although weekly ophthalmoscopic examinations are a part of our regular routine, the use of the ophthalmoscope is not essential for the detection of

Fig. 2 Photographs of typical experimental animals. Above—negative control that received the flavin-deficient diet only. Alopecia of the face and cataract are evident. Below—rat which received the flavin-deficient diet supplemented with 90 micrograms of flavin weekly. There was no evidence of alopecia or cataract. The animals were photographed on the seventy-fifth day of experiment, at which time they weighed 35 and 150 gm. respectively.

most cases of cataract. After dilation of the pupil with 0.5% atropine sulphate solution, most cases of early cataract may be detected without the use of the ophthalmoscope, if the eyes of such animals are observed in a partially darkened room with the aid of a small pocket flash lamp. The beam is directed into the eye at an oblique angle from the line of vision of the observer, and the lens opacity is seen as a snowy mass, appearing to be deep in the eye. Frequently the lens opacities are obscured by dense opacties in the cornea (keratitis); such

keratitis is frequently transient, however, and upon disappearing renders the cataract visible. Upon proceeding to maturity the cataract may be seen even in the presence of dense keratitis.

The eleven control animals survived between 49 and 87 days, with an average survival period of 72 days. In addition to exhibiting keratitis and cataract, many of them showed more or less extensive alopecia and roughness of hair. None of them developed the inflammation and exfoliation of the paws characteristic of 'florid' dermatitis or 'rat acrodynia.'

None of the eighteen animals receiving flavin exhibited any evidence of cataract; a few rats on the lowest flavin level showed alopecia and mild keratitis. Several developed ulcers about the head, but at no time did the flavin rats show the dermatitis characteristic of vitamin B_6 deficiency. The animals receiving flavin were killed at the end of 15 weeks with the exception of one rat on each level of feeding. The animal receiving 30 micrograms weekly died during the nineteenth week without showing cataract. The other two are still alive after 25 weeks without any evidence of lenticular opacities.

Since we were primarily interested in the cataract-preventive action of the flavin rather than a quantitative determination of its growth-promoting activity, the animals were not subjected to a preliminary depletion period before the feeding of flavin was started. Hence the growth curves may not be strictly comparable to growth curves obtained by investigators employing a depletion period. The growth-promoting action of the sample of natural flavin was essentially the same as that of the synthetic material.

Differentiation of the cataract-preventive factor (flavin) from the rat dermatitis-preventive factor. As was pointed out in the introduction, evidence from several laboratories has differentiated two relatively heat-stable vitamins of the B complex required by the rat. Evidence of a similar nature has been obtained in this laboratory, and since it also differentiates the cataract-preventive factor from the rat dermatitis-preventive vitamin, it is pertinent to the present discussion.

Four groups of rats were given a basal diet deficient in all the B vitamins (diet 625 without the rice polish extract) supplemented in various ways, as follows:

Group I. Deficient diet supplemented with 60 micrograms crystalline vitamin B (Merck) weekly. This was deficient in both flavin and the dermatitis-preventive factor (vitamin B_6).

Group II. Deficient diet supplemented with rice polish extract (diet 625). This diet was deficient in flavin.

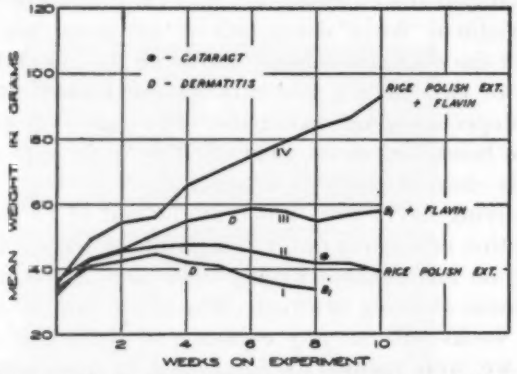

Fig. 3 Mean growth curves of groups of rats receiving a diet deficient in all B vitamins, supplemented as indicated at the ends of the curves. Rice polish ext., extract of rice polish prepared with 80% alcohol, as contained in diet 625; flavin, 30 micrograms lactoflavin weekly per rat; B_1, 60 micrograms crystalline vitamin B weekly per rat; D, dermatitis, i.e., inflammed paws, nose and ears; *, cataract. The roman numerals refer to the groups as discussed in the text.

Group III. Deficient diet supplemented with 60 micrograms crystalline vitamin B weekly and 30 micrograms lactoflavin weekly. This diet was deficient in the rat dermatitis-preventive factor (vitamin B_6).

Group IV. Deficient diet supplemented with rice polish extract and 30 micrograms lactoflavin weekly. This diet contained enough of all dietary essentials for fair growth; the growth-limiting factor was flavin.

Mean growth curves for these groups of rats are shown in figure 3; the roman numerals on the curves correspond to the four groups as indicated above. The animals in group I all

developed dermatitis (acrodynia) between the twenty-fifth and thirty-sixth days, with an average time of appearance of 29 days which is marked on the curve by the letter 'D.' Since this diet was deficient in flavin also, it would be expected that the rats receiving it would also develop cataract. Most of the rats died during the first 8 weeks of experiment, however, and only in the few remaining animals did eye changes develop. The animals in group II developed cataract, and the average time of appearance is shown on the curve by an asterisk. None of them developed the characteristic dermatitis of vitamin B_6 deficiency, although alopecia was commonly seen.

The animals in group III made only very slight growth, but all survived 10 weeks. These rats developed dermatitis but not cataract, as should be expected since they received the cataract-preventive substance (flavin), but did not receive any material containing the dermatitis-preventive vitamin. The animals in group IV did not show any evidence of either cataract or dermatitis, and made fair gains in weight. Growth was obviously limited by the rather low level of flavin feeding.

DISCUSSION AND CONCLUSIONS

The above data give evidence that our diet 625 is adequate for good growth when supplemented with pure lactoflavin. This would indicate that the rice polish extract in the diet contains considerabe amounts of vitamin B, the rat dermatitis-preventive factor (vitamin B_6), and any other of the B vitamins required for growth of the rat except flavin. Since we have not fed lactoflavin in quantities greater than 90 micrograms weekly, we do not yet know whether further increments in flavin feeding will result in further increments in growth. It may be that diet 625 does not contain enough of these various factors for normal growth, but it is clear that it contains enough of them for growth up to 10 gm. weekly. Using this diet with experiments where the growth is 10 gm. weekly or less, therefore, it is obvious that flavin is the limiting growth factor. We feel that these results justify the statement that this diet is suitable for the biological estimation

of flavin. Indeed, it is now possible to interpret all the assays for 'vitamin G' which we have reported in the last 6 years in terms of flavin, uncomplicated by the antidermatitis factor or other growth essentials.

Since a very large percentage of the rats given our flavin-deficient diet develop cataract, and cataract may effectively be prevented with pure flavin under rigidly controlled conditions, it is apparent that flavin is a specific cataract-preventive vitamin for the rat. We find no other adequate explanation for the data reported above. It has been the practice to refer to each of the vitamins in terms of the frank disease or syndrome resulting from marked deprivation of that substance, i.e., antineuritic, antiscorbutic, antirachitic, etc. In common with this practice of referring to the vitamins in terms of the most conspicuous pathological manifestations resulting from their lack, we believe that it is entirely appropriate to refer to flavin as the cataract-preventive vitamin. In doing so there is no implication that the only function of flavin is the prevention of cataract, any more than the term antiscorbutic implies that the prevention of scurvy is the only useful function of ascorbic acid. However, cataract is a specific condition not readily confused with the pathology of any other nutritional deficiency, and, prior to the death of the experimental animal, is probably the only irreparable result of flavin deficiency.

As has already been mentioned, a number of other laboratories have also reported cataract resulting from vitamin G deficiency, but some investigators have stated that they failed to observe the condition, and many more have made no mention of it whatever. Although it would be inappropriate for us to attempt to explain why certain other laboratories have failed to observe cataract as a result of vitamin G insufficiency, there are certain quite obvious reasons that may explain some such failures. It is probable that some investigators have been dealing primarily with a deficiency of some one or more of the growth essentials other than flavin in their vitamin G experiments. Also, a very small amount of flavin will delay

the appearance of cataract for a considerable period, and greatly reduce its incidence. Consequently a small amount of flavin as a contaminant of a diet component could prevent the appearance of cataract during an ordinary experimental period. For instance, Supplee, et al. ('36) have detected the presence of flavin in certain so-called 'vitamin-free' caseins, and have stated that some of the commonly used methods for rendering casein vitamin-free do not remove the last trace of flavin. Our own experience with various casein preparations, using production of cataract as an evidence of flavin deficiency, has led us to similar conclusions. Some of the conditions influencing the appearance of cataract in flavin deficiency are being investigated, and the results will be reported later.

Our results indicate that flavin is ineffective in preventing the dermatitis of so-called 'rat pellagra' or 'rat acrodynia,' a conclusion previously reached by several other investigators. This dermatitis, characterized by inflammation and edema of the extremities, must be regarded as a specific syndrome resulting from a deficiency of some vitamin which has not yet been identified chemically. Flavin deficiency, evidenced by alopecia and cataract, can now be clearly distinguished from this other symptom-complex.

SUMMARY

Young rats given a diet deficient in all B vitamins, but supplemented with an extract of rice polish prepared with 80% ethyl alcohol, developed cataract and alopecia. Litter mates receiving the same diet, further supplemented with 30 micrograms, 60 micrograms or 90 micrograms of lactoflavin weekly, did not exhibit cataract. From this it is concluded that flavin is a specific cataract-preventive substance for the rat. Growth was roughly proportional to the amount of flavin fed, and the highest level of feeding promoted good growth. None of the animals receiving the diet, either with or without flavin supplement, developed dermatitis of the extremities. It is obvious that the rice polish extract contained considerable amounts of vitamin B, the antidermatitis

factor (vitamin B_6), and any other of the B vitamins required by the rat except flavin.

Rats given a diet deficient in all B vitamins, supplemented with crystalline vitamin B only, developed the characteristic inflammation and edema of the extremities termed 'rat pellagra,' 'florid dermatitis' or 'rat acrodynia.' Flavin failed to prevent this syndrome. Thus flavin deficiency, evidenced by alopecia and cataract, can be clearly distinguished from this other symptom-complex.

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THE PROTEIN MINIMA FOR NITROGEN EQUILIBRIUM WITH DIFFERENT PROTEINS[1]

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FOUR FIGURES

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In the present study the determinations of protein minima for nitrogen equilibrium with different proteins were carried out as prerequisites for the proper evaluations of these foodstuffs with respect to their ability in promoting serum protein regeneration (Melnick, Cowgill and Burack, '36). By such a procedure it was possible to estimate with some degree of accuracy what fraction of the dietary protein, ingested by the dogs subjected to plasmapheresis, is utilized for the nitrogen metabolism of the organism as a whole. Theoretically the relative potencies of the various proteins for the regeneration of serum protein should then be evidenced solely by the effects obtained when the test increment quantities are fed. However, the determination of a protein minimum for nitrogen equilibrium is obviously also an indication of its biological value. For a summary of the nutritional values of many proteins the reader is referred to the review article by Boas-Fixsen ('34). It was hoped that an experimental determination of the minimum for nitrogen equilibrium of one of the proteins would suffice, with the aid of values reported by other investigators, to indicate what the corresponding

[1] These data form part of a dissertation presented by Daniel Melnick to the Graduate School, Yale University, for the degree of doctor of philosophy, June, 1936. The expenses of this investigation were defrayed by a grant from the Research Fund, Yale University School of Medicine.

amounts should be for the other proteins. However, the relative values reported in the literature were found to be so conflicting (see table 1, Boas-Fixsen, '34) that it was impossible with any degree of accuracy to apply these data to our investigations of serum protein regeneration. It was evident, therefore, that we should determine with our own dogs, fed according to our standardized technic, the minima for nitrogen equilibrium of the proteins to be tested for their respective potencies in the regeneration of serum protein.

It is not our purpose to cite here the extensive literature concerning the biological values of proteins in view of the fact that several review articles are available (Cathcart, '21; Mitchell, '24 b; Boas-Fixsen, '34).

PLAN OF STUDY

Investigations of the protein minima for nitrogen equilibrium were attempted, using in all cases artificial 'synthetic' diets, wherein the protein was the only dietary variable operating. In his review of this subject, Cathcart ('21) states: "that the search for an absolute minimum is like the search of the philosopher for absolute truth. There is not one minimum but many protein minima—the minimum is a resultant of many factors." These numerous factors may be listed as follows: a) the nature of the foodstuffs fed with the protein; b) the completeness of the diet, both qualitatively and quantitatively; c) the caloric value of the food given; d) the degree of maturity of the experimental animal; e) the activity of the experimental animal; f) the environmental temperature; g) the nutritive condition of the animal, and h) an adequate preliminary adjustment period. In the present study attempts have been made to control the above factors.

The dogs were fed 'synthetic' diets: the chemical nature and exact proportions of all the nutrients were known. The plan of the diet followed the kilo-unit scheme, as suggested by Cowgill ('23). The components of the ration were the protein under investigation, hydrogenated vegetable oil,[2]

[2] 'Crisco,' obtained from the Proctor and Gamble Co., N. Y.

sucrose, dextrin, bone ash, modified Osborne and Mendel salt mixture (Wesson, '32) and the vitamin adjuvants. This combination was used during the study on serum protein but was later modified in that the fat component was replaced by lard and the dextrin by sucrose. These modifications seemed to render the diet more appetizing to our dogs subjected to plasmapheresis, which procedure proved to affect adversely the urge to eat. The determination of the minimal amount of protein in the diet to maintain nitrogen equilibrium required from three to six experimental rations for each of the proteins tested. To arrive at this value it was necessary to begin with a distinctly negative balance and by repeated protein increments attempt to attain equilibrium. Such a procedure is essential since nitrogen equilibrium may occur at different levels in a normal animal provided the protein intake is above the amount minimal for nitrogen equilibrium (Voit, 1881). In all cases in the present study the protein was increased gradually at the expense of an isodynamic portion of the carbohydrate fraction, the other constituents of the diet remaining constant. Thus, the nature of the foodstuffs fed with the protein was the same throughout the investigations. A variation of this factor may well produce fallacious results in that another variable is then introduced, namely, the protein-sparing action of carbohydrates versus that of fat (Lusk, '28; Maignon, '33). A representative series of diets is given in table 1 to illustrate how the dietary factors were controlled in the present investigation.

In our study it was desirable that the vitamin adjuvants should not introduce any appreciable amount of protein. As sources of vitamins A and D, two tablets of a cod liver oil concentrate[3] were given daily per 5 to 7 kg. of body weight. These tablets contain no protein. For vitamin B(B_1) an extract of rice polishing[4] was administered. The sample contained 56 international units per gram and was given

[3] "White's Cod Liver Oil Concentrate Tablets," kindly furnished by the Health Products Corp., Newark, N. J.

[4] "Ryzamin," obtained from the Burroughs Wellcome Co., Tuckahoe, N. Y.

daily to the extent of 56 mg. per kilogram of body weight. This dosage of the vitamin is adequate and, although the extract contained 0.94% nitrogen, it introduced no appreciable source of dietary protein. As a source of vitamin G(B_2) liver extract

TABLE 1

Casein diets constructed on the kilo-unit plan and employed in the determination of the minimal amount of this protein for nitrogen equilibrium

FOODSTUFF	GRAMS	CALORIES ABSOLUTE	CALORIES	COMPOSITION	CALORIES PER GRAM OF DIET	NITROGEN CONTENT
			%	%		%
Casein [1]	1.36	4.55	5.7	8.41		
	(1.14)			(7.07)		
Lard	3.77	34.00	42.5	23.27	4.94	1.122
Sucrose	10.36	41.45	51.8	64.00		
Bone ash	0.40	....	....	2.47		
Salt mixture	0.30	....	....	1.85		
Total	16.19	80.00	100.0	100.00		
Casein	1.85	6.25	7.8	11.38		
	(1.56)			(9.58)		
Lard	3.77	34.00	42.5	23.21	4.92	1.521
Sucrose	9.94	39.75	49.7	61.10		
Bone ash	0.40	....	....	2.46		
Salt mixture	0.30	....	....	1.85		
Total	16.26	80.00	100.0	100.00		
Casein	2.34	7.9	9.85	14.35		
	(1.97)			(12.07)		
Lard	3.77	34.0	42.50	23.08	4.89	1.918
Sucrose	9.53	38.1	47.65	58.28		
Bone ash	0.40	....	....	2.44		
Salt mixture	0.30	....	....	1.85		
Total	16.34	80.0	100.00	100.00		
Casein	2.52	8.5	10.6	15.40		
	(2.12)			(12.96)		
Lard	3.77	34.0	42.5	23.02	4.88	2.058
Sucrose	9.38	37.5	46.9	57.31		
Bone ash	0.40	....	....	2.44		
Salt mixture	0.30	....	....	1.83		
Total	16.37	80.0	100.0	100.00		

[1] 13.37% nitrogen; 84.1% pure.

The numbers in parentheses are the absolute values.
The vitamin adjuvants are described in the text.

no. 343[5] was given daily in the dosage of 210 mg. per kilogram of body weight. Our sample of liver extract no. 343 contained 8.1% of nitrogen of which we estimate 45% may be regarded as potentially protein nitrogen as a result of the analytic data obtained by Kapeller-Adler and Luisada ('34). Thus, the source of vitamin G(B_2) yielded an appreciable amount of 'protein,' namely, 50 mg. per kilogram of body weight. At all the feedings the vitamin adjuvants[6] were administered apart from the food mixture, thus insuring an adequate vitamin intake by the animals.

The kilo-unit, as first described by Cowgill ('23) is designed to yield 80 calories. However, inasmuch as our dogs were kept in metabolism cages and their activity thus reduced, the daily administration of 70 calories per kilogram of body weight was found to be optimal for maintenance. The vitamin adjuvants yielded 1.5 calories per kilogram. With such a caloric intake the dogs maintained optimal and constant weights and always possessed hearty appetites at each feeding. The dogs were fed once each day, at as nearly the same hour as possible, this being between 9 and 10 A.M., and always consumed their ration within 5 to 10 minutes after it was offered. This feeding schedule was rigidly adhered to. Investigators (Krummacher, 1896; Gebhardt, 1897; v. Hoesslin and Lesser, '11) have repeatedly shown that if the food be distributed over the day and not concentrated in one meal, there tends to be a decrease in the amount of total nitrogen excreted. Chanutin and Mendel ('22), employing both low and high protein diets, were unable to observe any superior utilization of the dietary protein in dogs when subjected to fractional as compared with single feedings. Mitchell ('24 a) has confirmed these findings on rats fed a 5 to 10% protein diet, but reported a higher utilization of protein in the 10

[5] We are indebted to the Eli Lilly Co., Indianapolis, Ind., for a generous supply of this material in powdered form.

[6] It was found convenient to prepare an aqueous suspension of 75 gm. of liver extract no. 343 and 20 gm. of Ryzamin in 250 cc. of water. This suspension, adjusted to a pH just acid to litmus, was given orally in the dosage of 0.7 cc. per kilogram of body weight.

to 18% diets when the daily rations were administered at regular intervals throughout the day. In order to avoid any question on this point, this variable was controlled by maintaining the feeding technic constant throughout the study. Furthermore, soon after the ingestion of a protein meal, there is a rapid rise in the output of nitrogen in the urine resulting ultimately in the excretion of 70% of the ingested protein within 8 hours (Veraguth, 1897; Gruber, '01; Haas, '08; Pepper and Austin, '15). Thus by feeding once a day at regular time intervals, there will be no tendency for 'carry-overs' in the nitrogen elimination from the previous periods.

The experimental animals were adult female dogs. Inasmuch as growing organisms require proportionally greater and variable nitrogen intakes, nitrogen balance studies are conducted more satisfactorily upon mature animals. The dogs were housed in metabolism cages where their activities were limited and maintained as constant as possible. The environmental temperature was poorly controlled, being dependent to a considerable extent upon the season of the year. Thus, the temperature of the dog room varied for the most part from 70 to 80°F. Moderate muscular exercise (Campbell and Webster, '21; Wilson, Long, Thompson and Thurlow, '25) and the temperature of the environment (Youngburg and Finch, '26) have never been demonstrated to exert any definite effect on nitrogen metabolism. However, the control of these two factors is essential in regulating the caloric balance which, in turn, may affect the nitrogen balance. Preliminary to the investigations on each of the proteins, the dogs were given a vermifuge and then given an excess of vitamin B(B_1) to saturate the tissues with this dietary essential.[7] By preliminary feedings the weights of the animals were adjusted to an optimal nutritive condition, as estimated by the nutritive index formula (Cowgill, '28). The amounts of the diets fed, 70 calories per kilogram of body weight, were calculated on the basis of Cowgill's suggestion that a nutritive index value

[7] The source of the vitamin used here was 'Embo,' powdered wheat embryo, kindly furnished by the General Mills Inc., Minneapolis, Minn.

of 0.30 be regarded as the optimum. Throughout the experimental periods the greatest deviation of the dogs from this value was easily within ± 1.5%.

Preliminary to each of the collection periods, an adequate adjustment period is essential. Our most detailed study was conducted upon serum protein. Urinary nitrogen values were determined daily. Fecal nitrogens were determined over a 4-day period and averaged to give the daily aliquots. The values obtained indicate that a 5-day period is adequate under our experimental conditions for the organism to adjust itself to a new dietary regime. Subsequent studies on casein, lactalbumin, and gliadin were conducted with 5 days taken as the minimum for adjustment. The following 4-day urine samples were pooled and Kjeldahl determinations run in triplicate, the value obtained being translated to daily urinary nitrogens. A similar procedure was followed in determining the fecal nitrogens.

To insure as quantitative a collection of the excreta as possible, catheterizations were resorted to in the case of the urine samples, and charcoal was used to mark the feces. The urine was collected with toluene added as a preservative and sulfuric acid to prevent the volatilization of ammonia. Care was taken to wash the cages thoroughly each day, the washings being added to the urine. The feces were collected over the 4-day period and were preserved under acidified (sulfuric acid) alcohol. These were subsequently dehydrated on the steam bath by repeated evaporations of added alcohol. The dried product was ground, passed through a sieve to remove hair, and then analyzed for total nitrogen.

RESULTS

The serum protein minimum for nitrogen equilibrium

Beef serum was employed as the source of serum protein.

The blood was collected in oiled centrifuge bottles (500 cc.) and permitted to remain in the cold for 4 to 24 hours. The serum was removed by suction, the syneresis being hastened by centrifuging. The serum was then adjusted to pH 4.7

and added slowly to an equal volume of a boiling 20% NaCl solution, subjected to mechanical stirring. The coagulum was filtered, washed with hot water, and finally defatted and dehydrated by successive acetone and ether washings. The final product was air dried as 75 to 80°C. From 12 liters of blood 300 to 350 gm. of this crude serum protein could be obtained.

The nitrogen content of the preparation was 12.2% indicating 76.4% purity (Jones, '31). Moisture analyses of 16.00%, and sodium chloride determinations of 7.63% (Van Slyke and Sendroy, '23), accounted for the remaining 23.6% of impurity. No iron (Fe^{+++}) was present.

Six experimental periods, four on one dog and two on the other, were required to determine the minimal amount of serum protein in the diet capable of maintaining these dogs in nitrogen equilibrium. Four diets were employed. Adjusted to a nutritive index of 0.30, dog no. 8, a fox terrier, weighed 6.75 kg.; dog no. 1, a wire-haired terrier, 6.83 kg. In table 2 the results of these studies made with serum protein are summarized.

In the last column of table 2 are listed the data for several negative and two positive nitrogen balances. These values, expressed in terms of grams of nitrogen balance per kilogram of body weight at a nutritive index of 0.30, are plotted as ordinates in figure 1 against the per cent of calories furnished by the protein. By the method of least squares the average line, AB, is obtained giving equal weight to all points on the graph, i.e., of dogs no. 1 and no. 8. From the graph it is evident that the minimal amount of serum protein in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 8.5% of the caloric intake.

The casein minimum for nitrogen equilibrium

The sample of casein employed in this study was a commercial preparation,[8] which contained 13.37% nitrogen. This was indicative of an 84.1% purity (Jones, '31).

[8] Obtained from the Lister Bros., New York, N. Y.

TABLE 2

Determination of the serum protein minimum for nitrogen equilibrium

DOG NO.	EXPERIMENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	%	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
8	3	5.7	86.5	426	2.5	10	0.970	0.111	....	....	6.91	Adjustment
	1	5.7	86.5	426	2.5	10	0.970	0.111	1.290	0.162	6.80	—0.371 gm. N
	4	7.0	97	473	2.5	10	1.320	0.111	....	....	6.77	Adjustment
	4	7.0	97	473	2.5	10	1.320	0.111	1.430	0.180	6.70	—0.179 gm. N
	3	7.45	97	473	2.5	10	1.400	0.111	....	....	6.70	Adjustment
	4	7.45	97	473	2.5	10	1.400	0.111	1.460	0.181	6.66	—0.130 gm. N
	5	8.65	97	473	2.5	10	1.625	0.111	....	....	6.66	Adjustment
	2	8.65	97	473	2.5	10	1.625	0.111	1.538	0.189	6.61	+0.009 gm. N
1	5	7.45	98	478	2.5	10	1.415	0.111	....	....	6.71	Adjustment
	3	7.45	98	478	2.5	10	1.415	0.111	1.381	0.264	6.68	—0.119 gm. N
	5	8.65	98	478	2.5	10	1.640	0.111	....	....	6.72	Adjustment
	4	8.65	98	478	2.5	10	1.640	0.111	1.474	0.247	6.59	+0.030 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

Four experimental diets were employed in this study on four dogs. Dogs no. 1 and no. 8, used in the determination of the serum protein minimum, were no longer available. At a nutritive index of 0.30, dog no. 9, a spitz, weighed 8.00 kg.; dog no. 10, a fox terrier, 8.95 kg.; dog no. 11, a bulldog, 7.76 kg.;

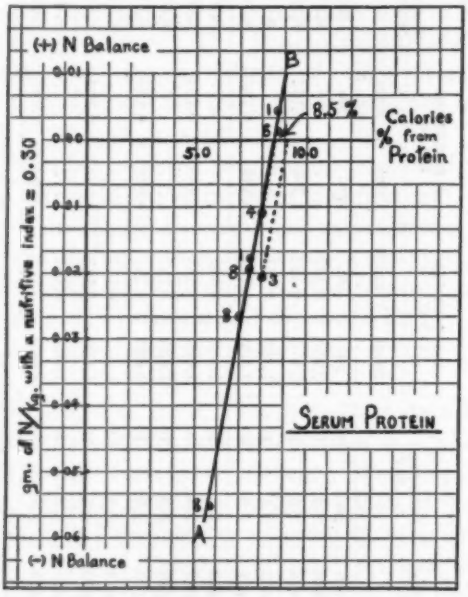

Fig. 1 The effect of increasing amounts of serum protein in the diet on the improvement in the negative nitrogen balance. The symbols, 1 and 8, refer to the dogs yielding the data from which the calculated curve, AB, was drawn. Symbols, 3 and 4, represent the experimental findings recorded with dogs no. 3 and no. 4,, respectively; the protein minima for these animals were estimated by the method described in the text (see dotted lines).

and dog no. 12, a mongrel, 5.81 kg. Dog no. 12 was later found to be a coprophagist, and therefore no feces were obtained for analyses. This animal ate the diet poorly, about one-fourth of the ration being fed forcibly daily; it also showed a steady loss of weight. The dog was subsequently discarded. In table 3, summarizing the balance studies, are included the

TABLE 3

Determination of the casein minimum for nitrogen equilibrium

DOG NO.	EXPERIMENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	%	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
9	5	5.7	113.5	560	3.0	12	1.274	0.140	....	....	8.00	Adjustment
	4	5.7	113.5	560	3.0	12	1.274	0.140	1.695	0.233	8.00	—0.514 gm. N
	5	7.8	114	560	3.0	12	1.735	0.140	....	....	8.10	Adjustment
	4	7.8	114	560	3.0	12	1.735	0.140	1.813	0.250	8.04	—0.188 gm. N
	5	10.6	114.5	560	3.0	12	2.360	0.140	....	....	8.21	Adjustment
	4	10.6	114.5	560	3.0	12	2.360	0.140	1.960	0.258	8.17	+0.282 gm. N
10	5	7.8	127.5	627	3.25	13	1.940	0.158	....	....	8.87	Adjustment
	4	7.8	127.5	627	3.25	13	1.940	0.158	2.370	0.305	8.76	—0.577 gm. N
	5	10.6	128.5	627	3.25	13	2.642	0.158	....	....	8.95	Adjustment
	4	10.6	128.5	627	3.25	13	2.642	0.158	2.583	0.300	8.91	—0.083 gm. N
11	5	9.85	111	543	3.0	12	2.129	0.135	....	....	8.09	Adjustment
	4	9.85	111	543	3.0	12	2.129	0.135	1.980	0.276	8.06	+0.008 gm. N
12	7	7.8	83	407	2.5	10	1.262	0.100	....	....	5.30	Adjustment
	4	7.8	83	407	2.5	10	1.262	0.100	1.800	†	5.22	—0.438 gm. N
	6	10.6	83.5	407	2.5	10	1.720	0.100	....	....	5.12	Adjustment
	4	10.6	83.5	407	2.5	10	1.720	0.100	1.630	†	5.06	+0.190 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

values from this animal, using the urinary nitrogen data which were available.

In figure 2, these data are plotted according to the same scheme as that used for figure 1. The curve, AB, was calculated by the method of least squares, employing the results

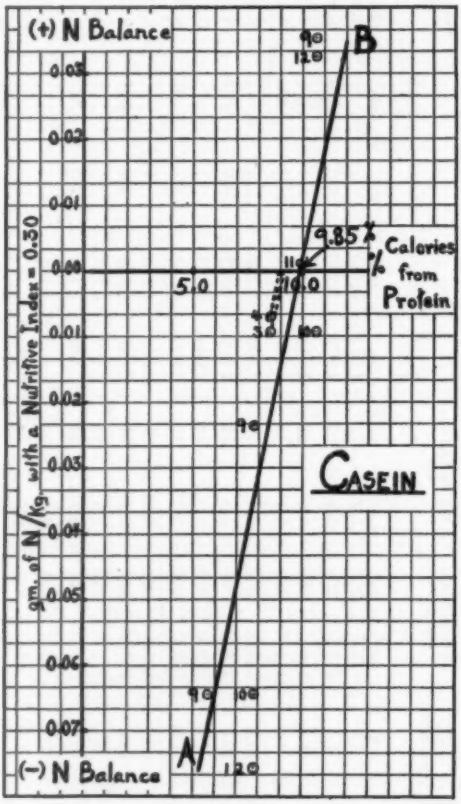

Fig. 2 The effect of increasing amounts of casein in the diet on the improvement in the negative nitrogen balance. The symbols, 9, 10 and 11, refer to the dogs yielding the data from which the calculated curve, AB, was drawn. Symbols 3 and 4, represent the experimental findings recorded with dogs no. 3 and no. 4, respectively; the protein minima for these animals were estimated by the method described in the text (see dotted lines).

obtained only from dogs no. 9, no. 10 and no. 11. From the figure it is evident that the minimal amount of casein in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 9.85% of the caloric intake.

The lactalbumin minimum for nitrogen equilibrium

The sample of lactalbumin employed in this study was a commercial preparation,[9] which contained 11.68% nitrogen. This was indicative of a 76.0% purity (Jones, '31).

Three experimental diets were employed in this study on three dogs. At a nutritive index of 0.30, dog no. 13, a mongrel, weighed 9.23 kg.; dog no. 9, 8.00 kg.; and dog no. 11, 7.76 kg. Table 4 summarizes the nitrogen balance studies made with lactalbumin as the protein.

In figure 3 the results are plotted, and a calculated curve, AB, drawn using the same scheme as that followed with the other proteins. From the figure, it is evident that the minimal amount of lactalbumin in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 6.75% of the caloric intake.

The gliadin minimum for nitrogen equilibrium

The gliadin employed in this study was obtained from gluten flour.[10] The method used in preparing the gliadin is that employed by Mr. L. S. Nolan of the Connecticut Agricultural Experiment Station. It gives large yields in relatively short periods when compared with the older methods. This procedure is described in detail in a communication from the experiment station (Nolan and Vickery, '36).

The nitrogen content of the preparation was 14.81%, indicating an 84.1% purity (Jones, '31). Gliadin prepared by the method mentioned above has been used in this laboratory in feeding experiments and has proven to be effective in stunting rats.

[9] Obtained from the National Milk Sugar Co., Bainbridge, N. Y.

[10] Obtained from the Battle Creek Food Co., Battle Creek, Mich. It is reported as 'containing 85% gluten.'

TABLE 4

Determination of the lactalbumin minimum for nitrogen equilibrium

DOG NO.	EXPERIMENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	%	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
13	5	5.7	131.5	645	3.5	14	1.410	0.162	....	....	9.47	Adjustment
	4	5.7	131.5	645	3.5	14	1.410	0.162	1.510	0.335	9.51	—0.273 gm. N
	5	6.5	132	645	3.5	14	1.608	0.162	....	....	9.53	Adjustment
	4	6.5	132	645	3.5	14	1.608	0.162	1.450	0.370	9.55	—0.050 gm. N
	7	7.45	132.5	645	3.5	14	1.840	0.162	....	....	9.46	Adjustment
	4	7.45	132.5	645	3.5	14	1.840	0.162	1.518	0.370	9.56	+0.114 gm. N
9	5	6.5	114.5	560	3.0	12	1.394	0.140	....	....	7.94	Adjustment
	4	6.5	114.5	560	3.0	12	1.394	0.140	1.243	0.231	7.98	+0.060 gm. N
	6	7.45	115	560	3.0	12	1.594	0.140	....	....	7.95	Adjustment
	4	7.45	115	560	3.0	12	1.594	0.140	1.305	0.240	7.94	+0.189 gm. N
11	6	7.45	112	543	3.0	12	1.560	0.135	....	....	8.02	Adjustment
	4	7.45	112	543	3.0	12	1.560	0.135	1.485	0.237	8.06	—0.027 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

It was noted that the dogs had some difficulty consuming the diet containing this protein due to the tendency for the gliadin to 'gum up' and stick to the teeth. The addition of an equal part of water to the diet produced a loose pasty mass which all the animals ate eagerly.

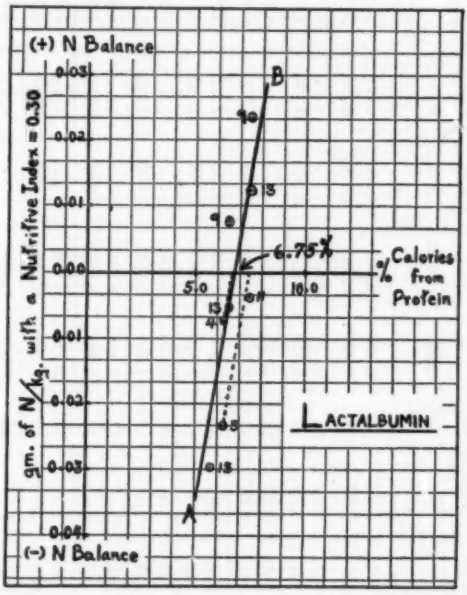

Fig. 3 The effect of increasing amounts of lactalbumin in the diet on the improvement in the negative nitrogen balance. The symbols, 9, 11 and 13, refer to the dogs yielding the data from which the calculated curve, AB, was drawn. Symbols 3 and 4, represent the experimental findings recorded with dogs no. 3 and no. 4, respectively; the protein minima for these animals were estimated by the method described in the text (see dotted lines).

When gliadin is fed as the sole protein, there is a deficiency with respect to the essential amino acid, lysine. Therefore, the length of the primary adjustment period was extended to 8 days. Subsequent adjustment periods were also greater than the usual 5 days as employed in the experiments with the other proteins. It is interesting to note here that Abder-

TABLE 5

Determination of the gliadin minimum for nitrogen equilibrium

DOG NO.	EXPERIMENTAL PERIOD	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
							Basal	Vitamins	Urine	Feces		
	days	%	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
10	8	7.45	127.5	627	3.25	13	2.060	0.158	....	....	8.96	Adjustment
	4	7.45	127.5	627	3.25	13	2.060	0.158	2.470	0.229	8.84	—0.481 gm. N
	6	10.6	128.5	627	3.25	13	2.930	0.158	....	....	8.90	Adjustment
	4	10.6	128.5	627	3.25	13	2.930	0.158	3.266	0.198	8.92	—0.376 gm. N
	6	22.0	131.5	627	3.25	13	6.068	0.158	....	....	8.91	Adjustment
	4	22.0	131.5	627	3.25	13	6.068	0.158	6.030	0.239	8.90	—0.043 gm. N
11	8	10.6	111	543	3.0	12	2.530	0.135	....	....	8.09	Adjustment
	4	10.6	111	543	3.0	12	2.530	0.135	2.778	0.186	8.11	—0.299 gm. N
	6	16.0	112.5	543	3.0	12	3.830	0.135	....	....	8.10	Adjustment
	4	16.0	112.5	543	3.0	12	3.830	0.135	3.950	0.190	8.10	—0.175 gm. N
	8	25.0	115	543	3.0	12	5.992	0.135	....	....	8.09	Adjustment
	4	25.0	115	543	3.0	12	5.992	0.135	5.830	0.180	8.08	+0.117 gm. N
9	8	13.0	115	560	3.0	12	3.206	0.140	....	....	7.98	Adjustment
	4	13.0	115	560	3.0	12	3.206	0.140	3.375	0.201	8.00	—0.230 gm. N
13	8	13.0	132.5	645	3.5	14	3.699	0.162	....	....	9.46	Adjustment
	4	13.0	132.5	645	3.5	14	3.699	0.162	3.905	0.256	9.40	—0.300 gm. N
	8	25.0	136.5	645	3.5	14	7.112	0.162	....	....	9.52	Adjustment
	4	25.0	136.5	645	3.5	14	7.112	0.162	6.846	0.258	9.50	+0.170 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

halden ('12) in his studies with amino acid mixtures corresponding in composition to various native proteins experimented with mixtures simulating gliadin and found 3 to 5 days to be adequate for adjustment.

Nine experimental periods were employed in this study using the same dogs as in the other proteins studied. Six

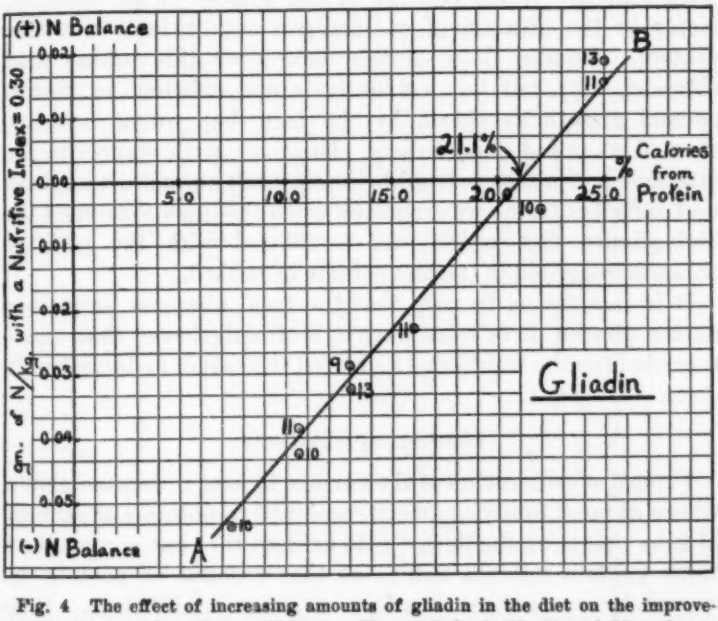

Fig. 4 The effect of increasing amounts of gliadin in the diet on the improvement in the negative nitrogen balance. The symbols, 9, 10, 11 and 13, refer to the dogs yielding the data from which the calculated curve, AB, was drawn.

experimental diets were employed. The summary of these nitrogen balance studies is given in table 5.

Figure 4, which is similar in construction to the other figures, is based on the data of table 5. From the figure it is evident that the minimal amount of gliadin in the diet capable of maintaining these normal dogs in nitrogen equilibrium under these conditions should furnish 21.1% of the caloric intake.

Peters and Van Slyke ('32) state that "any protein which is deficient in or entirely lacks one or more of these (essential) amino acids cannot be used alone to secure minimum nitrogen equilibrium. The amino acid deficiency becomes the limiting factor in the use of such a food" (p. 280).

Considering the results obtained in this study, it would seem that the lysine content of the gliadin is the limiting factor in the attainment of nitrogen equilibrium with this protein. Gliadin does contain 0.63% of this amino acid (Mendel, '23), but this evidently is not sufficient to permit normal growth as shown by the fact that young rats are stunted when fed gliadin diets. However, with respect to the attainment of nitrogen equilibrium in adult animals, it appears that sufficient lysine was ingested when the dogs consumed the high-gliadin diet which was associated with the positive balances recorded in the last column of table 5.

It is interesting to note that the fecal nitrogen excretions on the gliadin diets were always distinctly lower than those recorded for the same dogs when fed the casein or lactalbumin diets.

General applications

From a consideration of the various figures presented above, where at most four dogs were employed to obtain a maximum of nine points on the figure, it is evident that one cannot term these protein minima values as that amount of protein required by *every* dog to attain nitrogen equilibrium. It is apparent that many more animals must be used with each of the experimental periods to establish what may be regarded as a truly normal value having general significance. Furthermore, the application of these values as the protein minima without experimental confirmation on other dogs, particularly with reference to the animals subjected to plasmapheresis, could be justifiably criticized. For example, as exemplified by the casein figure (fig. 2) the values for one dog (see no. 9) may be as low as 8.5% and for another dog (no. 10) as high as 11.5%. However, the former animal shows a correspondingly

TABLE 6

Estimation of the protein minima for nitrogen equilibrium with dogs subjected to only one nitrogen balance study

DOG NO.	EXPERIMENTAL PERIOD	PROTEIN	CALORIES FROM PROTEIN	BASAL RATION PER DAY		VITAMINS PER DAY		N INTAKE PER DAY		N EXCRETION PER DAY		WEIGHT OF DOG[1]	PERIOD; N BALANCE
								Basal	Vitamins	Urine	Feces		
	days		%	*gm.*	*cal.*	*gm.*	*cal.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	
3	6	Serum protein	8.0	240	1169	6.25	25	3.720	0.296	....	....	16.91	Adjustment
	4		8.0	240	1169	6.25	25	3.720	0.296	3.837	0.521	16.90	—0.342 gm. N
	6	Lact-albumin	6.25	239	1169	6.25	25	2.804	0.296	....	....	16.95	Adjustment
	4		6.25	239	1169	6.25	25	2.804	0.296	2.928	0.560	16.94	—0.388 gm. N
	6	Casein	8.5	238	1169	6.25	25	3.946	0.296	....	....	17.01	Adjustment
	4		8.5	238	1169	6.25	25	3.946	0.296	3.808	0.588	17.10	—0.154 gm. N
4	6	Serum protein	8.0	249	1211	6.5	26	3.860	0.303	....	....	17.04	Adjustment
	4		8.0	249	1211	6.5	26	3.860	0.303	3.883	0.472	17.14	—0.192 gm. N
	6	Lact-albumin	6.25	248	1211	6.5	26	2.908	0.303	....	....	17.50	Adjustment
	4		6.25	248	1211	6.5	26	2.908	0.303	2.855	0.480	17.45	—0.124 gm. N
	6	Casein	8.5	247	1211	6.5	26	4.095	0.303	....	....	17.30	Adjustment
	4		8.5	247	1211	6.5	26	4.095	0.303	3.985	0.534	17.25	—0.121 gm. N

[1] These are the averages of the daily weighings during each of the experimental periods.

better utilization of casein at all levels of feeding as compared with the other dog. This indicates that within certain limits two dogs may differ as to their protein minimum, but that they tend to show a parallel consistency in their reactions to the various levels of dietary protein intake. This is obvious when one notes that the slopes of the figures indicating the responses of the dogs to the various levels of protein intake are remarkably parallel to each other. Thus, if we feed any dog one of the above proteins at a level which would be productive of a slightly negative balance, mere extrapolation to the line of nitrogen equilibrium by drawing a line parallel to

TABLE 7

Protein minima for nitrogen equilibrium

DOG NO.	PROTEIN	CALORIES FROM PROTEIN
		%
3	Serum protein	9.1
	Lactalbumin	7.4
	Casein	9.1
4	Serum protein	8.5
	Lactalbumin	6.65
	Casein	8.9

the calculated curve makes it possible to estimate the protein minimum for that animal by means of only *one* nitrogen balance study. The fact that the balance should be slightly negative is desirable in that any differences in the slopes of the dog's responses to the dietary protein from that of the calculated curve will be reduced to a minimum.

This scheme has been applied in evaluating the protein minima for our dogs to be subjected to plasmapheresis. At a nutritive index of 0.30 dog no. 3, a female hound, weighed 16.70 kg.; dog no. 4, a female collie, 17.30 kg. The gliadin value was not determined inasmuch as possible toxic manifestations were feared (Melnick, '36). In table 6 is found a summary of these nitrogen balance studies.

These data, when treated according to the scheme followed in preparing the various figures, were plotted on the corresponding charts. By extrapolating to the line of nitrogen equilibrium in accordance with the plan stated above, the protein minima presented in table 7 were obtained.

DISCUSSION AND CONCLUSIONS

The casein value of 9.85%, obtained with dogs no. 9, no. 10 and no. 11 appears to be somewhat too high. The values obtained with the dogs no. 3 and no. 4, which were later subjected to plasmapheresis, have been averaged with those yielded by the other dogs, arbitrarily allotting double weight to the latter values because more points were obtained for those animals. These calculations yield the following as the protein minima for nitrogen equilibrium, expressed in terms of the per cent of total ingested calories furnished by the protein in diets such as are described in this paper:

Lactalbumin	*Serum protein*	*Casein*	*Gliadin*
6.9%	8.6%	9.4%	21.1%

According to these figures the relative biological values of these proteins for the attainment of nitrogen equilibrium may be listed as follows, assigning to lactalbumin the value of 100:

Lactalbumin	*Serum protein*	*Casein*	*Gliadin*
100	80	73	33

When comparing these values with those reported in the literature (Boas-Fixsen, '34), it is pertinent to point out that most of the biological values recorded by others were obtained from experiments made on another species, namely, the rat. Furthermore, the criteria employed in these other studies have not been the same. In some cases the proteins have been evaluated with respect to their ability to promote growth; in other experiments the amounts of the proteins needed for maintenance were determined. The influence of various proteins upon fertility, lactation, longevity and stability of the nervous system has also been studied. Nitrogen balance studies have also been conducted with the rat,

the biological value of the protein being based upon the percentage of absorbed nitrogen that escaped excretion through the kidney. In listing the above relative values for the proteins studied in the present investigation, we emphasize that they represent only the respective abilities of these foodstuffs to satisfy the general nitrogen requirements of the dog as evidenced by the attainment of nitrogen equilibrium. Furthermore, it should be clearly understood that these minimal values are not to be regarded as reproducible unless the same experimental technique, as outlined in the present paper, is followed. This point seems obvious, since the minimal amount of a protein needed for nitrogen equilibrium is not a fixed constant but a resultant of the operation of many factors, as described above in the section on 'Plan of study.' Thus, by merely changing the experimental conditions, it is quite possible that large variations in these protein minimal values would be obtained, even with the same dogs used as the experimental animals. Our data would seem to gain in significance because of the fact that for the most part, each comparative test of these proteins under our fixed conditions was made on the same animal, and the relative ratings yielded by the different animals were essentially the same.

SUMMARY

a. Lactalbumin, serum protein, casein and gliadin have been evaluated with respect to the minimal amount of each in the diet essential for the dog in attaining nitrogen equilibrium. Expressed as percentage of total ingested calories furnished by the protein, these values have been found to be 6.9, 8.6, 9.4 and 21.1%, respectively. The corresponding relative biological values for these proteins were listed as 100, 80, 73 and 33.

b. A method for the estimation of the protein minimum for nitrogen equilibrium for any dog, employing only one experimental period, has been described.

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THE VITAMIN B AND VITAMIN C CONTENT OF MARINE ALGAE

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Marine algae are relatively simple, chlorophyllous plants which usually grow either under or partly under water. They are not differentiated into root, stem and leaf, and are reproduced by spores instead of seeds. The large marine algae are often given the common name of seaweeds, and are designated according to color as green, red and brown algae. Chlorophyl gives the characteristic green color to the green algae (Chlorophyceae). The green color of the chlorophyl is almost completely masked by a red pigment in the red algae (Rhodophyceae). A brown pigment is found in the brown algae (Phaeophyceae) in addition to the chlorophyl. The real basis for the division of the algae into these three subclasses is certain differences in their method of reproduction, but the colors correspond so closely with these differences in reproduction that it is usually possible to assign an alga to its proper subclass by its color.

Marine algae or seaweeds, together with phytoplankton, play the same role in the life and nutrition of the sea that land plants do in terrestrial life, and must therefore be expected to satisfy the nutritional requirements of much of marine life.

Although very little seaweed is eaten in America, in many countries, especially in the Orient, algae are consumed in large quantities. A summary of the utilization of edible algae is given in 'Marine Products of Commerce,' (Tressler,

'23). Seaweeds contain no starch and the complex carbohydrates which are present are not readily hydrolyzed by the human digestive enzymes.

The value of marine algae as a food is due mainly to the inorganic salts present and the vitamins. Seaweeds have long been used for the prevention of goiter and for the prevention and cure of scurvy.

Very little work has been reported upon the vitamin content of marine algae. Two varieties of Hawaiian edible algae, limu eleele (a species of Enteromorpha) and limu lipoa have been tested (Miller, '27) and found to be poor sources of vitamins B and C.

A number of algae were collected in the region of the San Juan Archipelago during June, July and August of two successive years. Some of the species collected are used as food by Indians of the Pacific coast, and many of the species are of the same type and genus as those eaten in various parts of the world. Vitamin B and vitamin C were determined on some of the algae collected.

VITAMIN B

For the determination of vitamin B the algae were cleaned, air dried in the shade, and stored to be tested during the winter months.

Vitamin B(B_1) was determined by feeding tests (Chase and Sherman, '31). The rats which were used were weaned at 28 days, placed in individual cages with screen bottoms, and given a basal diet as follows, with distilled water for drinking:

The basal diet

	%
Casein (extracted)	18
Autoclaved yeast	10
Osborn and Mendel salt mixture	4
Cod liver oil	1
Olive oil	9
Starch	58

In extracting the casein, 400 gm. were stirred with 1.5 liters of 60% alcohol for 30 minutes, then allowed to stand over night. The alcohol was removed in a hydraulic press at 5000

pounds per square inch. The extraction with 60% alcohol was repeated, and the casein stirred with 95% alcohol for 1 hour, pressed, and dried at room temperature. The autoclaved yeast was heated at 20 pounds pressure for 4 hours, and then tested for the absence of the heat labile factor measured as B(B_1) and the presence of the heat stable factor measured as G(B_2). The Osborn and Mendel salt mixture was modified by the addition of 0.161 gm. of hydrated copper sulfate for every 134.8 gm. of calcium carbonate used in the mixture. Negative controls were run, feeding only the basal diet and distilled water, on which growth ceased uniformly near the end of the second week. Positive controls were run

TABLE 1

The average weekly gain in weight, in grams, of experimental animals fed graded amounts of algae during a 4-week experimental period

GRAMS OF ALGAE FED PER RAT PER DAY	ALARIA VALIDA	ENTERO-MORPHA INTESTINALIS	LAMINARIA BULLATA	PORPHYRA NEREO-CYSTIS	PORPHYRA PER-FORATA	RHODY-MENIA PERTUSA	ULVA LACTUCA
0.1	....	....	....	—2.4	—1.6	....	—2.4
0.2	—1.6	—6.5	....	5.7	1.6	—1.7	....
0.3	—0.3	—3.5	—1.7	8.7	6.3	1.3	3.5
0.5	6.2	—1.6	3.1	....	17.3	4.3	12.0
0.6	....	....	6.0	....	....	....	....
0.8	11.5	....	....	....	....	10.0	....

by supplementing the basal diet with an alcoholic extract of wheat germ which had been shown to contain the heat labile factor and be free of the heat stable factor, normal growth was obtained in every case.

To determine the vitamin B(B_1) in marine algae, the animals were first depleted of vitamin B by feeding the basal diet until cessation of growth and the animals started to lose weight, which required about 2 weeks. The diet was then supplemented by graded amounts of the substance to be tested. The algae were ground and fed separately in weighed portions, which were consumed quantitatively. Both sexes of rats were used, and the litter mates and sexes distributed among the groups on different tests to give reliable results.

The results obtained are given in table 1.

The unit of vitamin B for each seaweed was calculated using the Sherman unit (Sherman, '32) which is defined as: "a unit of vitamin B may be taken as that amount which when fed daily under experimental conditions as described will induce in a standard test animal an average gain of 3 gm. per week during the test period." Table 2 gives the values obtained. The results on a good grade brewers yeast fed under the same conditions is given for comparison.

TABLE 2

The vitamin B content of marine algae

MATERIAL TESTED	WHERE OBTAINED	GRAMS FED PER DAY FOR A GAIN IN WEIGHT OF 3 GM. PER WEEK	UNITS (SHERMAN) OF VITAMIN B (B_1) PER GRAM
Phaeophyceae:			
Alaria valida	Upper sub-littoral zone	0.4	2.5
Laminaria sp.	Dredged from a depth of 5 to 10 fathoms	0.5	2.0
Rhodophyceae:			
Porphyra nereocystis	Surface	0.18	5.5
Porphyra perforata	Littoral zone	0.2	5.0
Rhodymenia pertusa	Dredged from a depth of 5 to 10 fathoms	0.45	2.2
Chlorophyceae:			
Enteromorpha sp.	Littoral zone	....	Trace only
Ulva lactuca	Surface	0.25	4.0
Dried brewers yeast	.....	0.075	13.3

From the data in table 2 it is shown that of the algae tested several are very good sources of vitamin B, comparing favorably with many common fruits and vegetables. Porphyra was found to be the best source of the algae tested and Enteromorpha the poorest source. Enteromorpha had only a trace of vitamin B present. No relation was found between the source of the algae, that is the depth from which it was obtained, and the vitamin B content.

VITAMIN C

Much of the seaweed collected for shipment to the market is dried and prepared in such a manner as to destroy any

vitamin C which might have been present; however, among peoples living on the coast fresh seaweeds have been an important part of the diet.

Vitamin C (ascorbic acid) was determined in the fresh algae, by titration with 2-6-dichlorophenolindophenol, using the method of Bessey and King ('33). The algae were blotted free of excess water with a clean towel or filter paper immediately before analysis.

Before applying a titration method such as that of Bessey and King to a new type of food product, the chemistry of which is as little known as the chemistry of the algae, it is first necessary to test the method to be sure that the reducing substance being measured is ascorbic acid. The validity of the determination was tested by two methods. The first method was the precipitation of interfering substances with mercuric acetate by the procedure of Emmerie and Van Eekelen ('34), which includes the use of hydrogen sulfide and would therefore insure the reduction of any oxidized ascorbic acid, if it were present in the oxidized form. The second was the determination of vitamin C by the method of Tauber and Kleiner ('35), using a specific oxidase for ascorbic acid.

The ascorbic acid was determined upon extracts of Porphyra perforata and Ulva lactuca, using the method of Emmerie and Van Eekelen ('34). The extract of the seaweed was treated with a solution of mercuric acetate to precipitate substances other than ascorbic acid which might reduce the dye used for titration. The precipitate was removed by centrifuging, and the supernatent liquid treated with hydrogen sulfide to remove the excess of mercury. The hydrogen sulfide was removed with nitrogen. Titrations were made on the original extract and the solution after precipitation. The results showed that 99% of the reducing power measured as vitamin C in Porphyra remained after precipitation and treatment with hydrogen sulfide, and 95% of the reducing power remained in the extract of Ulva. A sample of Rhodymenia which gave a titration indicating less than 0.01 mg. of vitamin C per gram gave the same results after treatment, indicating that the ascorbic acid was not present in the oxidized form.

Extracts of Porphyra perforata and Ulva lactuca prepared according to the method of Bessey and King ('33) and an extract of Porphyra treated with mercuric acetate and hydrogen sulfide according to the method of Emmerie and Van Eekelen ('34) were oxidized with a specific oxidase prepared according to the method of Tauber and Kleiner ('35), who state that the reducing substance removed may be considered as 'true' ascorbic acid.

Enzyme solutions were prepared by the extraction of both yellow crookneck squash, and summer squash. Both enzyme preparations gave 100% oxidation of the ascorbic acid of lemon juice in 30 minutes. The enzyme oxidized from 96% to 100% of the reducing power of Porphyra and 73% of the reducing power of Ulva. Controls were run using boiled enzyme preparation, and incubation without enzyme to check on the possibility of auto-oxidation. The results indicate that the reducing substance in algae measured by titration with 2-6-dichlorophenolindophenol is ascorbic acid.

Using the method of Bessey and King titrations were made upon a number of marine algae; the results given in table 3 are calculated to the blotted wet weight. Several samples of each species were analyzed and the average of the results reported.

Results obtained by titration of twenty-four samples of lemons obtained from the local market during the same period gave an average value of 0.43 mg. of vitamin C per gram of lemon juice, varying from 0.31 to 0.57 which is appreciably lower than the average value of 0.57 for lemon juice given by Bessey and King ('33). From the table it will be seen that some of the seaweeds are as high or even higher in vitamin C than lemons purchased on the local market at the same time of year.

While no hard and fast rule can be drawn, in general those forms collected from the littoral and upper sublittoral zones were higher in vitamin C than forms dredged from considerable depth. Algae dredged from depths of 5 to 10 fathoms were practically devoid of ascorbic acid. Two samples of

Rhodymenia which had no vitamin C when dredged were stored in a live box at the surface for several days and upon titration gave values of 0.07 and 0.08 mg. per gram of algae indicating that under certain conditions probably controlled principally by light Rhodymenia was able to form and store vitamin C.

TABLE 3

Name of algae	*Vitamin C milligrams per gram wet weight*	
	1934	1935
Phaeophyceae (brown algae):		
From the surface, littoral and upper sublittoral zones:		
Alaria valida	0.53	
Egregia menziesii	0.04	
Fucus evanescens	0.24	0.36
Hedophyllum sessile	0.21	
Macrocystis pyrifera	0.19	
Postelsia palmaeformis	0.09	
Dredged from a depth of 5 to 10 fathoms:		
Agarum fimbriatum	0.02	
Costaria costata	0.02	
Desmarestia munda	0.01	0.01
Laminaria bullata	0.02	0.01
Chlorophyceae (green algae):		
From the surface and littoral zone:		
Enteromorpha sp.	0.15	0.11
Ulva lactuca	0.46	0.38
Rhodophyceae (red algae):		
From the surface, littoral and upper sub-littoral zones.		
Gigartina papillata	0.41	0.25
Grateloupia Cutleriae	Less than 0.01	
Halosaccion glandiforme	0.13	
Iridaea sp.	0.26	
Porphyra naiadum	0.36	
Porphyra nereocystis	0.53	
Porphyra perforata	0.60	
Prionitis Lyallii	0.03	
Turnerella pacifica		0.09
Dredged from a depth of 5 to 10 fathoms:		
Agardhiella tenera	Less than 0.01	
Anatheca furcata	Less than 0.01	
Callophyllis sp.		Less than 0.01
Dasyopsis plumosa	Less than 0.01	
Hymenena sp.	Less than 0.01	
Opuntiella californica	Less than 0.01	
Polyneura latissima	Less than 0.01	Less than 0.01
Rhodymenia pertusa	Less than 0.01	Less than 0.01

The local species of Porphyra were found to be very good sources of vitamin C; they are used as food by the Indians of the region and of Alaska. Various other species of Porphyra are widely used in different parts of the world (Tressler, '23) under the names of red laver in England, sloke in Ireland, slack in Scotland and amanori in Japan, where large quantities of it are cultivated and some of the crop eaten fresh. Ulva sp., called green laver, oyster green and sea lettuce, is also a good source of ascorbic acid. Alaria sp. or murlins is gathered and eaten on the coast of Scotland, Ireland and Iceland; the local species proved to be a good source of vitamin C. While other varieties of algae such as Rhodymenia sp., known as dulse, dilling, dulling and water leaf, together with many of the deep-sea red algae: as, Agardhiella, Polyneura, Dasyopsis, etc., were very poor sources of ascorbic acid.

CONCLUSIONS

1. Six of the seven algae tested were found to be good sources of vitamin B, comparing favorably with many fruits and vegetables.
2. Species of Porphyra were found to be richest source of both vitamin B and vitamin C among the algae tested.
3. A few of the species of algae tested were found to be as rich a source of vitamin C as lemons bought on the local market.
4. Algae growing in the littoral zone or on the surface tend to be higher in vitamin C than algae which are dredged from a depth of 5 to 10 fathoms.

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THE MULTIPLE NATURE OF THE VITAMIN D OF FISH OILS [1]

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In the introductory paper of this series (Bills, '35) the available evidence on the multiple nature of vitamin D was brought forth, and the conclusion reached that vitamin D exists in at least six chemically distinct forms. A seventh and an eighth form were subsequently recognized (Bills, '36). It was shown that none of these has been identified with the vitamin D of fish oils, and that the latter may be additional to the forms artificially prepared.

Until recently it was generally assumed that the vitamin D of fish oils is a single substance. Its concentration was known to vary widely in the liver oils of different species, or even of a particular species, but that the vitamin varies in kind, as well as in amount, was not appreciated before the investigation of tuna liver oil by Bills, Massengale and Imboden ('34). In that work we showed that the liver oil of one of the tuna species was less antirachitic than cod liver oil, rat unit for rat unit, in chickens. In this respect it differed from halibut liver oil, which resembled cod liver oil to within the errors of assay.

That certain fish oils have approximately the same chicken effectiveness, per rat unit, as cod liver oil is not remarkable. It is, however, a coincidence that several of these should be among the first that were studied. Thus sardine body oil,

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which Bills ('27) found equal to cod liver oil in assays with rats, has for a number of years been replacing cod liver oil in poultry raising. Rygh ('35) was unable to recognize any difference in chicken effectiveness, per rat unit, in the vitamin D of seventeen species of fish. Haman and Steenbock ('36) found that sardine oil, as well as halibut liver oil and the liver oil of burbot (a fresh water cod), had about the same efficacy as cod liver oil, per rat unit, for chickens. Black and Sassaman ('36) found that the liver oils of cod, halibut, swordfish and Japanese mackerel were about equally effective on chickens, per rat unit.

In the work of Rygh ('35) and especially of Dols ('35, '36) the vitamin D of tuna liver oil was found to be as effective as that of cod liver oil, per rat unit, for chickens. It will be shown, however, that these workers dealt with a species of tuna different from the one used by us. Haman and Steenbock ('36) observed that a commercial, mixed species, tuna liver oil was 'somewhat less effective' on chickens, per rat unit, than the other oils which they studied. Black and Sassaman ('36) in repeated trials with three Japanese species of tuna (bluefin, striped and yellowfin) found that the liver oils were only from 40 to 63% as effective, rat unit for rat unit, as cod liver oil on chickens.

The present report gives the details of our original experiments with the liver oils of halibut and California bluefin tuna, together with studies made with the oils of numerous other species of fish.

PROCEDURE

The means for distinguishing the forms of the vitamin was the differential response of rats and chickens. The oils, suitably diluted in maize oil, were assayed with rats and again with chickens, and the relative effectiveness of the vitamin D for chickens, rat unit for rat unit, was expressed on the basis of cod liver oil = 100. This was the device employed by Massengale and Nussmeier ('30) and by other workers in distinguishing the vitamin D of irradiated ergosterol from that of cod liver oil.

The assays with rats were conducted essentially in accordance with the technic of Bills, Honeywell, Wirick and Nussmeier ('31). This was modified to the extent of including the use of a reference oil (a replica of the international standard) in parallel with the test oil, and of a correspondingly revised procedure for the calculation of probable error. In every assay at least twenty litter-mate pairs of rats were used in the final assembly, in addition to those used in exploratory assays above and below the critical level. Thus we obtained assays of unusual accuracy, the probable errors of which ranged from $\pm 5\%$ to $\pm 7\%$, inclusive of the probable error of the reference replica. The potencies were expressed in international units (I.U.) of vitamin D per gram of oil. Needless to say, the oils which had been assayed with rats were kept under conditions which permitted no measurable loss of potency before they were assayed with chickens. In some instances, as a double check, the oils were re-assayed with rats after the assays with chickens were completed.

The assays with chickens were conducted according to the quantitative method of Massengale and Bills ('36). It should be pointed out that this new method was designed particularly for the detection of small differences in antirachitic potency with the chicken, and that it is the only method employing this species which includes means of calculating probable error. At least ten chicks were used in the assay of each oil. The probable errors ranged from $\pm 8\%$ to $\pm 13\%$ of the number of I.U. of vitamin D found.

The vitamin A determinations were made photometrically, with the apparatus known as the Hilger Vitameter. After suitable corrections for irrelevant absorption, they are probably at least as accurate as bioassays for this vitamin.

The oils studied are described below. All but nos. 27, 29, 30 and 31 were rendered either in this laboratory or in the oil factory of our company. With these exceptions, all livers were shipped from the place of origin in the frozen state. The identity of each kind was confirmed by inspection. In the following list, care was taken to use the zoölogical names

favored by the leading authorities, but the present state of fish taxonomy is such that the choice was not in every case wholly satisfactory.

Order Heterosomata (flatfishes)

Oil no. 1. Halibut. Hippoglossus hippoglossus (H. stenolepis). The fish were caught in the Pacific Ocean between northern California and Unalaska. The oil represented 26,000 kg. of livers. No adulteration. Potency, 41,000 I.U. of vitamin A and 1400 I.U. of vitamin D (rat) per gram.

Oil no. 2. Round-nosed sole. California sole. Eopsetta jordani. The fish were caught in Puget Sound, near Seattle. One hundred and eighty-one kilograms of livers. No adulteration. Potency, 110,000 I.U. of vitamin A and 1100 I.U. of vitamin D (rat) per gram.

Order Percomorphi, series Scombriformes (scombroid fishes: the tunas)

Oil no. 3. Bluefin tuna (of California). Leaping tuna, Thunnus saliens. Formerly and still most commonly, but wrongly, designated T. thynnus. Pacific Ocean, off southern California. Laboratory sample of 2 kg. of livers. No adulteration. Potency, 84,000 I.U. of vitamin A and 46,000 I.U. of vitamin D (rat) per gram.

Oil no. 3-x. Unsaponifiable fraction of oil no. 3, concentrated further by elimination of the cholesterol. Potency, 770,000 I.U. of vitamin A and 800,000 I.U. of vitamin D (rat) per gram (some vitamin A lost in saponification).

Oil no. 7-a. Bluefin tuna (of New England). Sometimes locally known as horse mackerel. Thunnus secundodorsalis. More commonly designated T. thynnus, but probably not identical with the latter. Provincetown, Massachusetts. The laboratory sample of 8 kg. of livers was kindly contributed by Mr. Harden F. Taylor of the Atlantic Coast Fisheries Co. No adulteration. Potency, 80,000 I.U. of vitamin A and 16,000 I.U. of vitamin D (rat) per gram.

Oil no. 9. Bluefin tuna (of Japan). Oriental tuna. In Japanese, 'maguro.' Thunnus orientalis. Formerly confused by some authors with T. thynnus. Pacific Ocean, off Japan. Four thousand one hundred and ninety-six kilograms of livers. Probably no adulteration. Potency, 36,000 I.U. of vitamin A and 61,000 I.U. of vitamin D (rat) per gram.

Oil no. 12. Albacore. Long-finned tuna. In Japanese, 'binnaga.' Thunnus germo (Germo germo). Pacific Ocean, off Japan. Two thousand seven hundred and seventy-six kilograms of livers. No adulteration. Potency, 18,000 I.U. of vitamin A and 41,000 I.U. of vitamin D (rat) per gram.

Oil no. 13. Yellowfin tuna. Yellowfin albacore. Neothunnus macropterus. Pacific Ocean, off Mexico and Central America. Nine thousand and eighty kilograms of livers. No adulteration. Potency, 48,000 I.U. of vitamin A and 13,000 I.U. of vitamin D (rat) per gram.

Oil no. 14. Striped tuna. Oceanic bonito. Commonly called skipjack in California. Katsuwonus pelamis. Pacific Ocean, off Mexico. Four hundred and fifteen kilograms of livers. No adulteration. Potency, 43,000 I.U. of vitamin A and 58,000 I.U. of vitamin D (rat) per gram.

Oil no. 15. California bonito. Sometimes called skipjack in California. Sarda lineolata. Pacific Ocean, off southern California and northern Mexico. One thousand three hundred and one kilograms of livers. No adulteration. Potency, 120,000 I.U. of vitamin A and 50,000 I.U. of vitamin D (rat) per gram.

Oil no. 16. California mackerel. Pacific mackerel. Pneumatophorus diego. Pacific Ocean, off southern California. Two thousand nine hundred and ninety-three kilograms of livers. Perhaps slightly adulterated with the California horse mackerel, Trachurus symmetricus. Potency, 88,000 I.U. of vitamin A and 1400 I.U. of vitamin D (rat) per gram.

Order Percomorphi, continued (miscellaneous percomorphs)

Oil no. 17. Swordfish. Xiphias gladius. Atlantic Ocean, from Massachusetts to Nova Scotia. Two thousand eight hundred and six kilograms of livers. No adulteration. Potency, 250,000 I.U. of vitamin A and 14,000 I.U. of vitamin D (rat) per gram.

Oil no. 18. Black sea-bass. California jewfish. Stereolepis gigas. Pacific Ocean, off northern Mexico. One thousand six hundred and two kilograms of livers. No adulteration. Potency, 520,000 I.U. of vitamin A and 4300 I.U. of vitamin D (rat) per gram.

Oil no. 19. Cabrilla. Epinephelus analogus. Gulf of California, northern part. Nine hundred and forty-six kilograms of livers. No adulteration. Potency, 170,000 I.U. of vitamin A and 220 I.U. of vitamin D (rat) per gram.

Oil no. 20-a. White sea-bass. Cynoscion nobilis (Atractoscion nobilis). Pacific Ocean, off southern California. One thousand five hundred and ninety-five kilograms of livers. No adulteration. Potency, 55,000 I.U. of vitamin A and 6000 I.U. of vitamin D (rat) per gram.

Oil no. 21. Totuava. Eriscion macdonaldi. Gulf of California, northern part. Two thousand eight hundred and eighty kilograms of livers. No adulteration. Potency, 56,000 I.U. of vitamin A and 1200 I.U. of vitamin D (rat) per gram.

Order Cataphracti (rockfishes, etc.)

Oil no. 22. Sablefish. 'Black cod' (not a codfish). Anoplopoma fimbria. Pacific Ocean, from nothern California to Alaska. Sixteen thousand and thirty-eight kilograms of livers. No adulteration. Potency, 82,000 I.U. of vitamin A and 340 I.U. of vitamin D (rat) per gram.

Oil no. 23-a. 'Lingcod' (not a codfish). Ophiodon elongatus. Pacific Ocean, from northern California to Alaska. Seven thousand five hundred and sixty-three kilograms of livers. No adulteration. Potency, 160,000 I.U. of vitamin A and 840 I.U. of vitamin D (rat) per gram.

Oil no. 24. Bocaccio. Sebastodes paucispinis. Pacific Ocean, off California. Laboratory sample of 7 kg. of livers. No adulteration. Potency, 77,000 I.U. of vitamin A and 2000 I.U. of vitamin D (rat) per gram.

Oil no. 25. Chili-pepper. Sebastodes goodei. Pacific Ocean, off California. Laboratory sample of 1 kg. of livers. No adulteration. Potency, 150,000 I.U. of vitamin A and 270 I.U. of vitamin D (rat) per gram.

Order Jugulares

Oil no. 26. Wolffish. Ocean catfish. Anarhichas lupus. Atlantic Ocean, off Nova Scotia. Laboratory sample of 6 kg. of livers. No adulteration. Potency, 1300 I.U. of vitamin A and 19 I.U. of vitamin D (rat) per gram.

Order Euselachii

Oil no. 27. Basking shark. Cetorhinus maximus. Pacific Ocean, off southern California. The oil was obtained from Mr. J. F. Grundell of Monterey, who rendered it from a few kilograms of livers. Probably no adulteration. Potency, no detectable amount of vitamin A; about 4 I.U. of vitamin D (rat) per gram.

Order Tectospondyli

Oil no. 28. Dogfish. Squalus suckleyi. Pacific Ocean, off California. Laboratory sample of 3 kg. of livers. No adulteration. Potency, 23,000 I.U. of vitamin A and 16 I.U. of vitamin D (rat) per gram.

Order Anacanthini, family Gadidae (codfishes)

Oil no. 29. Pollack. Pollachius virens. Atlantic Ocean, off Maine. About 25,000 kg. of livers. Adulteration probably trivial, if any. Potency, 2800 I.U. of vitamin A and 110 I.U. of vitamin D (rat) per gram.

Oil no. 30. Hake. Urophycis chuss and U. tenuis. (Very similar species, perhaps intergrading.) Atlantic Ocean, off Maine. About 50,000 kg. of livers. Adulteration probably trivial, if any. Potency, 2300 I.U. of vitamin A and 130 I.U. of vitamin D (rat) per gram.

Order Isospondyli

Oil no. 31. Sardine. Sardinia caerulea. Pacific Ocean, off southern California. Poultry oil, manufactured from the entire fish, and from trimmings and entrails, including liver. About 65,000 kg. of fish. Adulteration trivial, if any. Potency, 2700 I.U. of vitamin A and 110 I.U. of vitamin D (rat) per gram.

FINDINGS AND DISCUSSION

The oils in the above list represent twenty-five species. They include a number of the most important vitamin sources, as well as several of merely theoretical interest. The potencies are not necessarily typical of the species, even when the amount of livers represented is large. For example, 26 metric tons of halibut livers were taken for oil no. 1, but they were all from fish caught in the summer months, when the oil yield is high and the potency low. The cod liver oil, with which the master curve of response was originally established, was described in the paper on technique (Massengale and Bills, '36). In the present work, another cod liver oil of specially determined potency was administered from time to time, to make certain that the response of the chicks was normal.

The experimental findings are summarized in table 1. The table shows for each oil the number of I.U. of vitamin A and

TABLE 1

Relative effectiveness of vitamin D from different sources for rats and chickens

OIL NO.	NAME OF FISH (OR STEROL)	VITAMINS GIVEN PER 100 GM. DIET			RESPONSE OBTAINED			EFFICACY RATIO	
		A	D		Femur ash, %	I.U. of D/100 gm.	PE, %	C.L.O. = 100	PE, %
		I.U.	I.U.	PE, %					
1	Halibut	510	17.5	±7	45.42	15.1	+9; −9	86	+11; −11
2	Round-nosed sole	1900	18.9	6	45.57	15.4	9; 9	81	11; 11
3	Tuna, bluefin (Cal.)	13	7.2	7	35.98	...[1]	.....[1]	..[1]	.....[1]
3	Tuna, bluefin (Cal.)	110	57.6	7	42.55	9.3	8; 8	16	11; 11
3-x	Tuna, bluefin (Cal.)	41	42.4	7	41.03	7.4	9; 8	17	11; 11
3-x	Tuna, bluefin (Cal.)	81	84.9	7	45.92	16.5	8; 8	19	11; 11
7-a	Tuna, New England	39	7.9	7	39.99	6.4	10; 10	81	12; 12
7-a	Tuna, New England	46	9.2	7	41.45	8.0	9; 9	87	11; 11
9	Tuna, oriental	10	17.0	7	43.58	11.0	10; 9	65	12; 11
9	Tuna, oriental	30	51.0	7	46.55	18.7	10; 9	37	12; 11
12	Albacore	9	20.5	5	44.42	12.6	9; 9	61	10; 10
12	Albacore	9	20.5	5	44.82	13.5	9; 9	66	10; 10
12	Albacore	27	61.4	5	46.80	19.7	10; 9	32	11; 10
13	Tuna, yellowfin	40	10.7	7	45.23	14.6	9; 9	136	11; 11
14	Tuna, striped	7	9.6	5	35.62	...[1]	.....[1]	..[1]	.....[1]
14	Tuna, striped	43	56.5	5	45.12	14.3	9; 9	25	10; 10
15	California bonito	20	8.4	6	34.31	...[1]	.....[1]	..[1]	.....[1]
15	California bonito	140	58.0	6	45.93	16.5	10; 9	28	12; 11
16	California mackerel	630	9.8	7	44.01	11.8	10; 9	120	12; 11
17	Swordfish	180	10.1	6	45.53	15.3	9; 9	151	11; 11
18	Black sea-bass	930	7.8	7	41.98	8.5	9; 8	109	11; 11
19	Cabrilla	8600	11.0	7	40.83	7.2	9; 9	65	11; 11
20	White sea-bass	25	2.8	7	42.19	8.8	9; 8	314	11; 11
20	White sea-bass	55	6.0	7	45.74	15.9	9; 9	265	11; 11
21	Totuava	470	10.0	7	34.90	...[1]	.....[1]	..[1]	.....[1]
21	Totuava	2800	59.8	7	44.47	12.7	9; 9	21	11; 11
22	Sablefish	1600	6.8	7	43.59	11.0	10; 9	162	12; 11
22	Sablefish	2300	9.8	7	45.64	15.6	9; 9	159	11; 11
23-a	'Lingcod'	3000	15.8	6	45.66	15.7	9; 9	99	11; 11
24	Bocaccio	330	8.7	6	44.46	12.7	9; 9	146	11; 11
25	Chili-pepper	4200	7.2	7	40.01	6.4	10; 10	89	12; 12
26	Wolffish	680	9.9	7	43.08	10.1	10; 9	102	12; 11
27	Basking shark	nil	5.2	7	41.94	8.4	9; 8	162	11; 11
28	Dogfish	5800	4.0	7	42.50	9.2	9; 9	230	11; 11
29	Pollack	210	8.5	7	38.12	4.3	13; 13	51	15; 15
30	Hake	160	8.7	6	43.91	11.6	10; 9	133	12; 11
31	Sardine	270	10.7	7	43.96	11.7	10; 9	109	12; 11
Control	Cod	310	10.0	2	42.97	9.9	10; 9	99	10; 9
Control	Cod	250	8.0	2	42.08	8.6	9; 8	108	9; 8
Control	Cod	250	8.0	2	40.89	7.3	9; 9	91	9; 9
Control	Cod	250	8.0	2	42.05	8.6	9; 8	108	9; 8
Control	Cod	250	8.0	2	41.53	8.0	9; 9	100	9; 9
Control	Cod	130	4.0	2	38.81	5.1	11; 11	128	11; 11
Control	Maize oil	0	0.0	0	35.12	0.0	.....[1]	..[1]	.....[1]
Sterol	Irr. ergosterol	0	200.0	..[2]	40.00	6.4	.....[2]	3.2	.....[2]
Sterol	Irr. ergosterol	0	403.0	..[2]	43.30	10.4	.....[2]	2.6	.....[2]
Sterol	Irr. ergosterol	0	1800.0	..[2]	46.50	18.5	.....[2]	1.0	.....[2]
Sterol	Irr. cholesterol (ordinary, from spinal cord)	0	8.0	7	41.72	8.2	9; 8	103	11; 11
Sterol	Irr. 7-dehydrocholesterol	0	13.0	6	43.93	11.7	10; 9	90	12; 11

[1] Response too low for significant interpretation.

[2] Values taken from master curve of response (Massengale and Bills, '36).

vitamin D administered per 100 gm. of ration. It records the average femur ash percentage of the group of chicks which received the oil, and the number of I.U. of vitamin D per 100 gm. of ration which theoretically would have been required to produce the observed calcification if the vitamin D had been that of cod liver oil. Finally, it shows the efficacy ratio, on the basis of cod liver oil = 100, of the vitamin D that was actually administered. Probable errors, expressed as per cent of the found values, are given for the rat assays, the chick assays,[2] and the efficacy ratios. The values for the latter were calculated from the formula,

$$\text{Efficacy ratio error} = \sqrt{\text{rat error}^2 + \text{chick error}^2}$$

In most instances, the efficacy ratio error amounted to about 11%.

At the beginning of this discussion, attention must be called to a fundamental difficulty which is encountered in attempts to give numerical expression to the efficacy ratio, or relative effectiveness, of one form of vitamin D in terms of another, rat unit for rat unit in chickens. Inspection of the master curves for cod liver oil and irradiated ergosterol, given in our paper on the assay technic with chickens, discloses that the efficacy ratio is not a constant quantity; it varies with the degree of calcification produced. Thus, one kind of vitamin D may be 80% as effective as another, rat unit for rat unit in chickens, when a certain degree of calcification is involved, and 120% when a different degree of calcification is involved. Instances of this effect are seen in table 1, where the same oil was administered at two levels, and materially different efficacy ratios were obtained. The significance of all this is, that the efficacy ratios are valid only for the degree of calcification indicated.

The first oil studied was halibut liver oil. Its efficacy ratio was 86, which means that, rat unit for rat unit, this oil was

[2] The probable error for an assay with chicks varies with the kind of vitamin D being assayed (Massengale and Bills, '36). In table 1 it is calculated as it would be if the vitamin D were always that of cod liver oil.

86% as effective as cod liver oil for producing the observed degree of femur calcification (45.42% ash) in the chicken. If we accept the conventional standard of significance, that values differing by less than three times their probable error are not significantly different, we must admit that the observed difference is not significant. Another flatfish, the round-nosed sole, gave approximately the same efficacy ratio, 81, as halibut.

Halibut liver oil is essentially a rich source of vitamin A, but it is only a few times more potent than cod liver oil in vitamin D. It occurred to us that if different kinds of vitamin D exist in nature, a good place to look for them would be in the tuna liver oils which are enormously potent in vitamin D. We examined a sample of the oil from the California bluefin tuna, Thunnus saliens. This was administered to chicks at a level of 7.2 I.U. per 100 gm. of ration, a level sufficient to produce good calcification if the vitamin D were that of cod liver oil or halibut liver oil. The resulting femur ash was so low that significant interpretation of it was impossible. Then the dosage was increased eightfold and the efficacy ratio was found to be 16.

From the fact that certain esters of calciferol are inactive, or only slightly active in healing rickets before they are saponified, it seemed possible that the anomalous behavior of the tuna liver oil was due to the existence of its vitamin D as an ester or other complex which was easily hydrolyzable by the rat but difficultly hydrolyzable by the chicken. We therefore prepared the unsaponifiable fraction of this oil, assayed it with rats, and administered it to chicks. The efficacy ratio, at two levels of administration, was 17 and 19. It follows that the low relative effectiveness of the tuna liver oil was not due, at least not in large part, to the existence of its vitamin D in unavailable form.

After the preliminary announcement of the findings on tuna liver oil (Bills, Massengale and Imboden, '34), we were informed by Dr. A. L. Emmett of Parke, Davis and Company and by Mr. Harden F. Taylor of the Atlantic Coast Fishing

Company that in their experiences the chicken efficacy ratio of tuna liver oils was not low. The recent studies of Dols ('35, '36) and Rygh ('35) also indicated that the vitamin D of tuna liver oil compared well with that of cod liver oil, rat unit for rat unit with chickens. Upon inquiry it developed that the oil studied by Emmett was from mixed species of tuna, and the oil studied by Taylor came from Massachusetts tuna. The vitamin D concentrates studied by Dols and by Rygh were from European tuna.

It happens that the name Thunnus thynnus has until recently been applied to big tunas the world over. This was the designation we accepted for the California bluefin tuna in our preliminary note, and it was based on general usage as exemplified by Jordan and Evermann (1896, '02), Jordan ('25), Ulrey and Greeley ('28), Walford ('31) and others. However, Jordan and Evermann ('26) and Jordan, Evermann and Clark ('30) have in recent years changed their original view that the genus Thunnus is represented by only one species, T. thynnus. They now provisionally recognize eight species, of which we are concerned with the California T. saliens, the New England T. secundodorsalis, the Japanese T. orientalis, and the European T. thynnus. Knowledge of the tunas is so imperfect that this reclassification ventured by Jordan and Evermann ('26) on the basis of slight differences in photographs and drawings has not met with general acceptance by ichthyologists. However, our findings strongly support the reclassification, and incidentally introduce a new, biochemical basis for taxonomy.

The New England tuna, Thunnus secundodorsalis, lost its identity under T. thynnus in Jordan and Evermann (1896, '02), Jordan ('25) and Bigelow and Welsh ('25). It was later distinguished by Jordan and Evermann ('26) and Jordan, Evermann and Clark ('30) as above indicated. The livers of this species yielded an oil which contained approximately the same amount of vitamin A as the oil of the California bluefin. However, the vitamin D content was much lower than that of any California bluefin tuna liver oil that

we have encountered in the examination of many large and small samples. The outstanding difference, as shown in table 1, was that the efficacy ratio of the vitamin D was high, 84 —essentially in agreement with the private experiments of Mr. Taylor.

We have not had the opportunity to examine the liver oil of the European tuna, Thunnus thynnus, but if the findings of Dols ('35, '36) and Rygh ('35) can be accepted, this species possesses vitamin D similar in kind to that of T. secundodorsalis.

The Japanese bluefin tuna, 'maguro,' Thunnus orientalis, was T. thynnus in Jordan and Evermann (1896) and Jordan, Tanaka and Snyder ('13). It was recognized as a separate species by Kishinouye ('23) and Jordan and Evermann ('26). The characteristic feature of its vitamin content is that the effectiveness of its vitamin D, rat unit for rat unit on chickens, is between that of T. saliens and T. secundodorsalis. At two levels of administration, efficacy ratios of 65 and 37 were obtained. These are in agreement with the findings of Black and Sassaman ('36).

The albacore, or long-finned tuna, is Thunnus germo in the critical study by Kishinouye ('23); Germo germo in most of the check lists. The data in table 1 indicate that the chicken effectiveness of albacore liver oil, per rat unit, is similar to that of oriental tuna. Early in our work, we once observed an unusually low efficacy ratio, 12, in albacore liver oil. In that instance, however, there was some doubt as to the composition of the test sample. The findings were therefore not included in the table.

Leaving the genus Thunnus, we now consider the data for related tunas and other scombroid fishes. The vitamin D of the liver oil of yellowfin tuna, Neothunnus macropterus, had an efficacy ratio of 136, which appears to be significantly greater than the ratio 100 for cod liver oil. Black and Sassaman ('36) found a value of 63 for yellowfin tuna, but it is to be noted that their livers were from Japanese fish, whereas ours were from fish caught off the coast of Mexico

and Central America. The difference may be one of environment, or possibly of species.

The liver oils of Mexican striped tuna, Katsuwonus pelamis, and the closely similar California bonito, Sarda lineolata, failed to produce mentionable calcification in chickens when a rat unitage was administered that should have done well had the vitamin D been that of cod liver oil. At much higher levels, efficacy ratios of 25 and 28 were obtained. Black and Sassaman ('36) reported a ratio of 46 for Japanese striped tuna. The California mackerel, Pneumatophorus diego, is a small relative of the tunas. Its liver oil showed an efficacy ratio of 120, which is not significantly above the ratio 100 for cod liver oil. Black and Sassaman's similar findings for Japanese mackerel cannot be compared with ours, because Japanese mackerel is definitely a different species.

The liver oils of five miscellaneous percomorphs, all important new vitamin sources, were next investigated. Swordfish gave an efficacy ratio of 151, distinctly higher than that reported by Black and Sassaman ('36). Black sea-bass, Stereolepis gigas, which shares with its Asiatic cognate, Stereolepis ishinagi, the distinction of being the richest known source of vitamin A, gave an efficacy ratio of 109. Cabrilla, Epinephelus analogus, which is classed in the same family (Epinephelidae) with the black sea-bass, gave an efficacy ratio of 65. White 'sea-bass,' Cynoscion nobilis (which properly is not a sea-bass), gave an efficacy ratio of around 300, the highest observed in any of the twenty-five species which were compared with cod. Totuava, which is classed in the same family (Otolithidae) with the white sea-bass, gave an efficacy ratio of only 21.

The liver oils of four fish in the order Cataphracti were investigated. Sablefish liver oil at two levels of administration to chicks gave efficacy ratios of 162 and 159. Lingcod gave 99. Bocaccio and chili-pepper gave 146 and 89, respectively.

Our study was completed with six other oils from species in five zoölogical orders. The liver oil of wolffish rated 102.

The basking shark, which is an extraordinarily poor source of vitamins, gave an efficacy ratio of 162. Dogfish rated 230. Two codfishes, whose liver oils contribute materially to the 'cod liver oil' of certain localities, were investigated. The pollack, Pollachius virens, rated 51. The hake, Urophycis chuss and U. tenuis (species so similar that few fishermen can distinguish them), rated 133. Liver oils of the pollack and these particular hakes are legally cod liver oil; the difference in efficacy ratios would suggest that the potency of cod liver oil, as ordinarily expressed after assay with rats, is not an infallible measure of the effectiveness of cod liver oil for chickens. The oil of the sardine, Sardinia caerulea, was the only oil studied which was not wholly a liver oil. The specimen was a typical commercial sardine oil, enormous quantities of which are used in the poultry industry. We found the efficacy ratio to be 109, a value not significantly different from the value 100 for cod liver oil and quite in keeping with the experience of practical poultrymen.

In coming to the conclusion that the data summarized in table 1 demonstrate the existence of two or more kinds of vitamin D in fish oils, we point out the following. The number of species of fish investigated was large. In an ample number of instances the differences observed were much greater than the probable errors of assay. The extreme difference was between the oils of California bluefin tuna and the so-called white sea-bass, the latter being about eighteen times as effective for chickens, per rat unit, as the former. Even with allowance for the difficulty already mentioned in regard to the numerical evaluation of efficacy ratios, and the necessarily indirect comparisons via cod liver oil, the difference observed between the liver oils of white 'sea-bass' and California bluefin tuna is far greater than the probable errors involved.

Inspection of the efficacy ratio column in table 1 reveals no clustering of values around 100. Were it otherwise, i.e., if there were numerous values near 100, and gradually fewer above and below 100, one would suspect that an overlooked source of error was at work. As it is, the scatter of values

seems wholly erratic, and indicative of the likelihood that in a larger series of fish oils, even greater differences in relative effectiveness would be observed.

The possibility of suppressed availability of the vitamin D in certain oils would seem to have been discounted by the experiment with the unsaponifiable fraction of the first tuna oil. The possibility that free fatty acids in the oils played any role is eliminated by the fact that most of the oils, including those of highest and lowest relative efficacy, were practically neutral. Moreover, most of the oils were greatly diluted before they were mixed with the diet, so that the vehicle of the vitamin was principally maize oil. Lastly, it is obvious from table 1 that there is no relation between the vitamin A content of the oils and the efficacy ratio of their vitamin D.

Our findings are explicable on the basis that two (or more than two) kinds of vitamin D exist in fish oils, the proportions varying in the different oils. It now seems unlikely that any particular fish oil, such as cod liver oil, contains one kind exclusively. No fish oil has been found with as low an efficacy ratio as irradiated ergosterol (calciferol). The latter has an efficacy ratio of from 1.0 to 3.2 when administered at dosages sufficient to produce degrees of femur calcification comparable with those given by the fish oils under the conditions of our experiments. It is conceivable, therefore, though not especially probable, that calciferol is the component of the fish oil vitamin D complex which has the lower efficacy ratio. At the other extreme, no artificial kind of vitamin D has been found with as high an efficacy ratio as certain fish oils, particularly as white sea-bass liver oil.

In collaboration with Dr. F. G. McDonald we have undertaken to determine the efficacy ratios of all the known artificial forms of vitamin D. It is especially desired to ascertain whether any of these has as high an efficacy ratio as white sea-bass liver oil. If none has, then the component of the fish oil vitamin D complex which has the higher efficacy ratio will have to be regarded as a new form of vitamin D—the ninth,

in fact. So far, we have discovered that irradiated 7-dehydrocholesterol is the artificial form of vitamin D with the highest efficacy ratio. For the production of certain degrees of calcification in the chick it is as effective, rat unit for rat unit, as cod liver oil. Irradiated ordinary cholesterol is similar in efficacy ratio to irradiated 7-dehydrocholesterol. Irradiated sterols of the higher plants, and also, according to McDonald's findings, irradiated 22-dihydroergosterol, are distinctly lower in efficacy ratio than these, but higher than irradiated ergosterol. Details of our studies with the above-mentioned irradiated sterols, and of still other forms of vitamin D, will be given later.

SUMMARY

The liver oils of twenty-five species of fish were assayed in comparison with cod liver oil on rats and chickens. Rat unit for rat unit, some resembled cod liver oil, several were definitely less effective, and a few were more effective than cod liver oil. The oils which were relatively the least effective were those from bluefin tuna of California, oriental tuna, striped tuna, bonito, albacore and totuava. The relatively most effective oil was that from the white sea-bass of California. The maximum observed difference in relative effectiveness was about eighteen times, a difference much greater than the probable errors of assay.

As possible causes of the differences, the following were eliminated: Existence of vitamin D in conjugated forms of unequal availability, synergism or antagonism of vitamin A, presence of free fatty acids, and the nature of the oily vehicle. It was concluded that the differences were due to the existence of two or more forms of vitamin D in the oils.

The possible relation of the fish oil vitamins D to certain artificial forms, particularly irradiated ergosterol and irradiated 7-dehydrocholesterol, was discussed. The relative effectiveness of irradiated ergosterol was lower than that of any fish oil. The relative effectiveness of irradiated 7-dehydrocholesterol was about the same as that of cod liver oil or irradiated ordinary cholesterol, but inferior to that of white sea-bass liver oil.

Oils of related species, particularly the several tunas, differed widely in relative effectiveness. The findings support the view that the big tunas formerly classed as Thunnus thynnus actually comprise several species, of which we have studied T. saliens, T. orientalis and T. secundodorsalis.

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